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SEARCH REQUEST FORM

2-175

Requestor's Name: Nancy Johnson Serial Number: 08/602,272
Date: 2.12.97 Phone: 305-5860 Art Unit: 1806

Search Topic:

Please write a detailed statement of search topic. Describe specifically as possible the subject matter to be searched. Define any terms that may have a special meaning. Give examples or relevant citations, authors, keywords, etc., if known. For sequences, please attach a copy of the sequence. You may include a copy of the broadest and/or most relevant claim(s).

Please search:

- Antibodies to tumor necrosis factor (TNF)
also called anti-TNF
- Methods of treating thrombotic disorder (stroke, ischemic event, thromboembolic disorder, cardiovascular disorder, heart attack, myocardial infarction, thrombophlebitis, cerebrovascular disorder) with antibodies to tumor necrosis factor.

- Please search authors =
Michael J. Elliott,
Ravinder N. Maini, or
Marc Feldman

and above subject matter.

- Please return results on 3 1/2" disc.

Thanks! Nancy J.

02/16/96

METHODS OF PREVENTING OR TREATING CARDIOVASCULAR, CEREbrovascular and Thrombo
DISORDERS WITH Tumor NECROSIS FACTOR ANTAGONISTS

STAFF USE ONLY

Date completed: 2/27/97
Searcher: IRVING
Terminal time: 84
Elapsed time: _____
CPU time: _____
Total time: 144
Number of Searches: 1
Number of Databases: 2

Search Site

☒ STC
☒ CM-1
☐ Pre-S

Type of Search

☐ N.A. Sequence
☐ A.A. Sequence
☐ Structure
☒ "

Vendors

☒ IC
☒ STN
☐ Dialog
☐ APS
☐ Geninfo
☐ SDC
☐ DARC/Questel

WEST**End of Result Set**

Generate Collection

Print

odp w
2nd ref.

L5: Entry 3 of 3

File: PGPB

Jan 24, 2002

PGPUB-DOCUMENT-NUMBER: 20020010180
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020010180 A1

09/754, 604

TITLE: TNFalpha antagonists and methotrexate in the treatment of TNF-mediated disease

PUBLICATION-DATE: January 24, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Feldmann, Marc	London		GB	
Maini, Ravinder N.	London		GB	

US-CL-CURRENT: 514/250; 424/145.1, 514/350

CLAIMS:

1-~~4~~
23

What is claimed:

1. A method for treating or preventing a tumor necrosis factor-mediated disease in an individual in need thereof comprising co-administering methotrexate and a TNF.alpha. antagonist to said individual, in therapeutically effective amounts.
2. A method of claim 1 wherein said TNF.alpha. antagonist and methotrexate are administered simultaneously.
3. A method of claim 1 wherein said TNF.alpha. antagonist and methotrexate are administered sequentially.
4. A method of claim 1 wherein the tumor necrosis factor-mediated disease is selected from the group consisting of: autoimmune disease, acute or chronic immune disease, inflammatory disease and neurodegenerative disease.
5. A method of claim 4 wherein said TNF.alpha. antagonist is administered in multiple doses.
6. A method of claim 1 wherein said TNF.alpha. antagonist prevents or inhibits TNF.alpha. synthesis or TNF.alpha. release.
7. A method of claim 6 wherein said TNF.alpha. antagonist is a phosphodiesterase inhibitor.
8. A method of claim 7 wherein said phosphodiesterase inhibitor is selected from the group consisting of: pentoxifylline and rolipram.
9. A method of claim 6 wherein said TNF.alpha. antagonist is selected from the group consisting of: thalidomide and tenidap.
10. A method of claim 6 wherein said TNF.alpha. antagonist is selected from the group consisting of: a A2b adenosine receptor agonist and a A2b adenosine receptor

enhancer.

11. A method of claim 5 wherein said TNF.alpha. antagonist is an anti-TNF.alpha. antibody or antigen-binding fragment thereof.

12. A method of claim 11 wherein said anti-TNF.alpha. antibody or antigen-binding fragment is a chimeric antibody or chimeric fragment, wherein said chimeric antibody or chimeric fragment comprises a non-human variable region specific for TNF.alpha. or an antigen-binding portion thereof and a human constant region.

13. A method of claim 12 wherein said chimeric antibody binds to one or more epitopes included in amino acid residues set forth in SEQ ID NO:1 or SEQ ID NO:2.

14. A method of claim 13 wherein said chimeric antibody competitively inhibits binding of TNF.alpha. to monoclonal antibody cA2.

15. A method of claim 13 wherein said chimeric antibody is monoclonal antibody cA2.

16. A method of claim 11 wherein said anti-TNF.alpha. antibody is a humanized antibody or antigen-binding fragment thereof.

17. A method of claim 16 wherein said humanized antibody binds to one or more epitopes included in amino acid residues set forth in SEQ ID NO:1 or SEQ ID NO:2.

18. A method of claim 11 wherein said anti-TNF.alpha. antibody is a resurfaced antibody or antigen-binding fragment thereof.

19. A method of claim 18 wherein said resurfaced antibody binds to one or more epitopes included in amino acid residues set forth in SEQ ID NO:1 or SEQ ID NO:2.

20. A method of claim 5 wherein said TNF.alpha. antagonist is a soluble TNF.alpha. receptor or functional portion thereof.

21. A method of claim 20 wherein said soluble TNF.alpha. receptor is selected from the group consisting of: p55 TNF.alpha. receptor and p75 TNF.alpha. receptor.

22. A method of claim 20 wherein said soluble TNF.alpha. receptor is a TNF.alpha. receptor multimeric molecule.

23. A method of claim 20 wherein said soluble TNF.alpha. receptor is a TNF.alpha. receptor immunoreceptor fusion molecule.

24. A method for treating or preventing arthritis in an individual in need thereof comprising co-administering methotrexate and a TNF.alpha. antagonist to said individual, in therapeutically effective amounts.

25. A method of claim 24 wherein said TNF.alpha. antagonist and methotrexate are administered simultaneously.

26. A method of claim 24 wherein said TNF.alpha. antagonist and methotrexate are administered sequentially.

27. A method of claim 24 wherein said TNF.alpha. antagonist is administered in multiple doses.

28. A method of claim 24 wherein said TNF.alpha. antagonist prevents or inhibits TNF.alpha. synthesis or TNF.alpha. release.

29. A method for treating or preventing rheumatoid arthritis in an individual in need thereof comprising co-administering methotrexate and a TNF.alpha. antagonist to said individual, in therapeutically effective amounts.

30. A method of claim 29 wherein said TNF.alpha. antagonist and methotrexate are administered simultaneously.

31. A method of claim 29 wherein said TNF.alpha. antagonist and methotrexate are administered sequentially.

32. A method of claim 29 wherein said TNF.alpha. antagonist is administered in multiple doses.

33. A method of claim 29 wherein said TNF.alpha. antagonist prevents or inhibits TNF.alpha. synthesis or TNF.alpha. release.

34. A method for treating or preventing Crohn's disease in an individual in need thereof comprising co-administering methotrexate a TNF.alpha. antagonist to said individual, in therapeutically effective amounts.

35. A method of claim 34 wherein said TNF.alpha. antagonist and methotrexate are administered simultaneously.

36. A method of claim 34 wherein said TNF.alpha. antagonist and methotrexate are administered sequentially.

37. A method of claim 34 wherein said TNF.alpha. antagonist is administered in multiple doses.

38. A method of claim 34 wherein said TNF.alpha. antagonist prevents or inhibits TNF.alpha. synthesis or TNF.alpha. release.

11. A method of claim 1, wherein the autoimmune disease is rheumatoid arthritis.
12. A method of claim 1, wherein the CD4+ T cell inhibiting agent is an antibody to T cells or to T cell receptors.
13. A method of claim 1, wherein the CD4+ T cell inhibiting agent is an antibody to an antigen presenting cell or to the receptors of an antibody presenting cell.
14. A method of claim 1, wherein the CD4+ T cell inhibiting agent is a peptide or small molecule which inhibits T cell interaction with antigen presenting cells.
15. A method of treating autoimmune or inflammatory disease in a mammal comprising administering to said mammal a therapeutically effective amount of a combination of a CD4+ T cell inhibiting agent and an inflammatory mediator which down-regulates cytokines.
16. A method of claim 15, wherein the inflammatory mediator is agent interfering with the activity or synthesis of TNF.
17. A method of claim 15, wherein the inflammatory mediator is an agent interfering with the activity or synthesis of IL-1.
18. A method of claim 15, wherein the inflammatory mediator is an agent interfering with the activity or synthesis of IL-6.
19. A method of claim 15, wherein the inflammatory mediator is a cytokine with anti-inflammatory properties.
20. A method of treating autoimmune or inflammatory disease in a mammal, comprising administering to said mammal a therapeutically effective amount of a combination of anti-CD4 antibody and anti-TNF antibody.
21. A method of treating autoimmune or inflammatory disease in a mammal, comprising administering to said mammal a therapeutically effective amount of a combination of anti-CD4 antibody and soluble TNF receptor.
22. A method of treating autoimmune or inflammatory disease in a mammal, comprising administering to said mammal a therapeutically effective amount of a combination of anti-CD4 antibody and TNF receptor/IgG fusion protein.
23. A method of treating autoimmune or inflammatory disease in a mammal, comprising administering to said mammal a therapeutically effective amount of a combination of cyclosporin A and anti-TNF antibody.

WEST**End of Result Set**

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L1: Entry 1 of 1

File: USPT

Jun 25, 2002

US-PAT-NO: 6410588

DOCUMENT-IDENTIFIER: US 6410588 B1

TITLE: Use of cannabinoids as anti-inflammatory agents

DATE-ISSUED: June 25, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Feldmann; Marc	London			GB
Malfait; Anne-Marie	Surrey			GB
Gallily; Ruth	Jerusalem			IL
Mechoulam; Raphael	Jerusalem			IL

US-CL-CURRENT: 514/454; 514/456, 514/719

CLAIMS:

What is claimed is:

1. A method of treating a patient suffering from rheumatoid arthritis comprising the step of administering to the patient a pharmaceutically effective amount of cannabidiol.
2. A method of treating a patient suffering from multiple sclerosis comprising the step of administering to the patient a pharmaceutically effective amount of cannabidiol.
3. A method of treating a patient suffering from ulcerative colitis comprising the step of administering to the patient a pharmaceutically effective amount of cannabidiol.
4. A method of treating a patient suffering from Crohn's disease comprising the step of administering to the patient a pharmaceutically effective amount of cannabidiol.
5. The method as in any one of claims 1-4, wherein the cannabidiol is combined with an anti-inflammatory compound.
6. The method as in any one of claims 1-4, wherein the cannabidiol is combined with a pharmaceutically acceptable carrier.
7. The method as in any one of claims 1-4, wherein the effective amount to be administered is between 1 .mu.g/kg/day to 50 mg/kg/day of patient body weight.

WEST



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L5: Entry 1 of 3

File: PGPB

PGPUB-DOCUMENT-NUMBER: 20030064070

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030064070 A1

TITLE: Multiple administrations of anti-TNF antibody

PUBLICATION-DATE: April 3, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Feldmann, Marc	London	PA	GB	
Maini, Ravinder Nath	London		GB	
Woody, James N.	West Chester		US	

US-CL-CURRENT: 424/145.1

CLAIMS:

What is claimed is:

1. A method of treating reoccurrence of a TNF-mediated disease in an individual having the TNF-mediated disease comprising administering multiple treatment cycles of an anti-TNF.alpha. antibody to said individual, each treatment cycle, other than the first, is administered once loss of response to the previous treatment cycle has occurred.
2. The method of claim 1 wherein the antibody is administered prophylactically.
3. The method of claim 1 wherein the TNF-mediated disease is selected from the group consisting of autoimmune disease, acute or chronic immune disease, bacterial infection, viral infection, parasitic infection, inflammatory disease, neurodegenerative disease, malignancy and alcohol-induced hepatitis.
4. The method of claim 1 wherein the anti-TNF antibody has an affinity for TNF.alpha. of at least about $K_a = 1 \times 10^8$ M.⁻¹.
5. The method of claim 4 wherein the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody or a resurfaced antibody or antigen binding fragment thereof.
6. The method of claim 5 wherein the antibody binds to one or more epitopes included in amino acid residues of about 87-108 (SEQ ID NO: 2) or about 59-80 (SEQ ID NO: 1) of hTNF.alpha..
7. The method of claim 5 wherein the antibody competitively inhibits binding of TNF.alpha. to monoclonal antibody A2.
8. The method of claim 5 wherein the antibody is a chimeric antibody.
9. The method of claim 8 wherein the antibody binds to one or more epitopes included in amino acid residues of about 87-108 (SEQ ID NO: 2) or about 59-80 (SEQ ID NO: 1) of hTNF.alpha..

10. The method of claim 8 wherein the antibody competitively inhibits binding of TNF.alpha. to monoclonal antibody cA2.

11. The method of claim 10 wherein the antibody is cA2.

12. A method of treating reoccurrence of rheumatoid arthritis in an individual in need thereof comprising administering to said individual multiple treatment cycles of an anti-TNF.alpha. antibody, each treatment cycle, other than the first, is administered once loss of response to the previous treatment cycle has occurred.

13. The method of claim 12 wherein the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody or a resurfaced antibody or antigen binding fragment thereof.

14. The method of claim 13 wherein the antibody binds to one or more epitopes included in amino acid residues of about 87-108 (SEQ ID NO: 2) or about 59-80 (SEQ ID NO: 1) of hTNF.alpha..

15. The method of claim 13 wherein the antibody competitively inhibits binding of TNF.alpha. to monoclonal antibody A2.

16. The method of claim 13 wherein the antibody is a chimeric antibody.

17. The method of claim 16 wherein the antibody binds to one or more epitopes included in amino acid residues of about 87-108 (SEQ ID NO: 2) or about 59-80 (SEQ ID NO: 1) of hTNF.alpha..

18. The method of claim 16 wherein the antibody competitively inhibits binding of TNF.alpha. to monoclonal antibody cA2.

19. The method of claim 18 wherein the antibody is cA2.

20. A method of treating a TNF-mediated disease in an individual having the TNF-mediated disease comprising administering multiple treatment cycles of an anti-TNA.alpha. antibody to said individual, each treatment cycle, other than the first, is administered once loss of response to the previous treatment cycle has occurred.

Venous Thrombosis

The formation or presence of a thrombus within a vein.

Thromboembolism

Obstruction of a vessel by a blood clot that has been transported from a distant site by the blood stream.

Purpura, Thrombotic Thrombocytopenic

A disease characterized by thrombocytopenia, hemolytic anemia, bizarre neurological manifestations, azotemia, fever, and thromboses in terminal arterioles and capillaries.

Coronary Thrombosis

Presence of a thrombus in a coronary artery, often causing a myocardial infarction.

L10 ANSWER 7 OF 8 USPATFULL on STN

ACCESSION NUMBER: 95:80223 USPATFULL

TITLE: DNA encoding a chimeric polypeptide comprising the extracellular domain of TNF receptor fused to IgG, vectors, and host cells

INVENTOR(S): Beutler, Bruce A., Dallas, TX, United States
Peppel, Karsten, Dallas, TX, United States
Crawford, David F., Irving, TX, United States

PATENT ASSIGNEE(S): Board of Regents, The University of Texas System,
Austin, TX, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5447851		19950905	<--
APPLICATION INFO.:	US 1992-862495		19920402 (7)	<--
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Draper, Garnette D.			
ASSISTANT EXAMINER:	Carlson, K. Cochrane			
LEGAL REPRESENTATIVE:	Arnold, White & Durkee			
NUMBER OF CLAIMS:	25			
EXEMPLARY CLAIM:	1			
NUMBER OF DRAWINGS:	10 Drawing Figure(s); 9 Drawing Page(s)			
LINE COUNT:	1479			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

PI	US 5447851	19950905	<--
AI	US 1992-862495	19920402 (7)	<--

AB . . . heavy chain. The invention relates as well to uses of the chimeric polypeptide, including: use as a reagent for the **antagonism** and assay of **TNF** and lymphotoxin from diverse species; use as a means of determining the mechanism by which **TNF**, or analogs thereof, interacts. . .

SUMM . . . also causes neutrophils to adhere to lung capillaries enhancing thrombus formation. This may result variously in disseminated intravascular coagulation, migratory **thromboses** and hemorrhagic necroses. **TNF**-alpha is also a potent endogenous pyrogen affecting hypothalamic neurons and promoting **IL-1** production. **TNF** has also. . .

L60 ANSWER 8 OF 14 USPATFULL on STN

ACCESSION NUMBER: 97:26904 USPATFULL

TITLE: Non-crosslinked protein particles for therapeutic and diagnostic use

INVENTOR(S): Yen, Richard C. K., Glendora, CA, United States

PATENT ASSIGNEE(S): Hemosphere, Inc., Irvine, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5616311		19970401
APPLICATION INFO.:	US 1994-212546		19940314 (8) <--
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1993-69831, filed on 1 Jun 1993, now abandoned And Ser. No. US 1992-959560, filed on 13 Oct 1992, now patented, Pat. No. US 5308620 which is a continuation-in-part of Ser. No. US 1991-641720, filed on 15 Jan 1991, now abandoned		
DOCUMENT TYPE:	Utility		
FILE SEGMENT:	Granted		
PRIMARY EXAMINER:	Lovering, Richard D.		
LEGAL REPRESENTATIVE:	Townsend & Townsend & Crew		
NUMBER OF CLAIMS:	26		
EXEMPLARY CLAIM:	1,26		
LINE COUNT:	2585		
CAS INDEXING IS AVAILABLE FOR THIS PATENT.			
AI	US 1994-212546	19940314 (8)	<--
DETD	Protein A,B,C,G,S; Ricin A; Proadifen (SKF-525A)1 Taxol; Thiolytes; Thiostrepton; Thrombin Thrombocytin; beta-Thromboglobulin; Thrombospondin ; Transferrin (apo-, partial iron, holo); Tumor Necrosis factor ; Vitronectin, Forskolin, Integrins; caged compounds (caged ATP, caged INsP3, caged cAMP, caged cGMP, caged GTP, caged carbamoyl chorine); Mezerein; Plasminogen;		

L56 ANSWER 18 OF 26 USPATFULL on STN

ACCESSION NUMBER: 95:47750 USPATFULL

TITLE: TNF inhibitors

INVENTOR(S): Christensen, IV, Siegfried B., Philadelphia, PA, United States

Esser, Klaus M., Downingtown, PA, United States

Simon, Philip L., Randolph, NJ, United States

PATENT ASSIGNEE(S): SmithKline Beecham Corp., Philadelphia, PA, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5420154		19950530	<--
	WO 9015534		19901227	<--
APPLICATION INFO.:	US 1992-852180		19920330 (7)	<--
	WO 1991-US5350		19910729	<--
			19920330	PCT 371 date
			19920330	PCT 102(e) date
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1990-562761, filed on 3 Aug 1990, now abandoned			
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Springer, David B.			
LEGAL REPRESENTATIVE:	Dinner, Dara L., Venetianer, Stephen, Lentz, Edward T.			
NUMBER OF CLAIMS:	8			
EXEMPLARY CLAIM:	1			
LINE COUNT:	726			
CAS INDEXING IS AVAILABLE FOR THIS PATENT.				
PI	US 5420154		19950530	<--
	WO 9015534		19901227	<--
AI	US 1992-852180		19920330 (7)	<--
	WO 1991-US5350		19910729	<--
			19920330	PCT 371 date
			19920330	PCT 102(e) date

DETD . . . experimental cerebral malaria (ECM) that reproduces some features of the human disease was prevented in mice by administration of an anti-TNF antibody. [See, Grau et al., Imm. Review 112:49-70 (1989)]. Levels of serum TNF correlated directly with the severity of disease and. . . Diseases. The deposition of silica particles leads to silicosis, a disease of progressive respiratory failure caused by a fibrotic reaction. **Antibody to TNF** completely blocked the silica-induced lung fibrosis in mice [See Piguet et al., Nature, 344:245-247 (1990)]. High levels of TNF production. . . blood flow [See, Vedder et al., PNAS 87:2643-2646 (1990)]. TNF also alters the properties of endothelial cells and has various pro-coagulant activities, such as producing an increase in tissue factor pro-coagulant activity and suppression of the anticoagulant protein C pathway as well as down-regulating the expression of thrombomodulin [See, Sherry et al., J. Cell Biol. 107:11269-1277 (1988)]. TNF also has pro-inflammatory activities which together with its early production. . . an inflammatory event) make it a likely mediator of tissue injury in several important disorders including but not limited to, myocardial infarction, stroke and circulatory shock. Of specific importance may be TNF-induced expression of adhesion molecules, such as intercellular adhesion molecule (ICAM). . .

L10 ANSWER 2 OF 8 PCTFULL COPYRIGHT 2003 Univentio on STN
 ACCESSION NUMBER: 1993006128 PCTFULL ED 20020513
 TITLE (ENGLISH): **TNF ANTAGONIST PEPTIDES**
 TITLE (FRENCH): PEPTIDES ANTAGONISTES DU FACTEUR DE NECROSE TUMORALE
 INVENTOR(S): RATHJEN, Deborah, Ann;
 WIDMER, Fred
 PATENT ASSIGNEE(S): PEPTIDE TECHNOLOGY LIMITED;
 RATHJEN, Deborah, Ann;
 WIDMER, Fred
 LANGUAGE OF PUBL.: English
 DOCUMENT TYPE: Patent
 PATENT INFORMATION:

NUMBER	KIND	DATE
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WO 9306128	A1	19930401
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DESIGNATED STATES

W: AT AU BB BG BR CA CH CS DE DK ES FI GB HU JP KP KR LK
 LU MG MN MW NL NO PL RO RU SD SE US AT BE CH DE DK ES
 FR GB GR IE IT LU MC NL SE BF BJ CF CG CI CM GA GN ML
 MR SN TD TG

APPLICATION INFO.: **WO 1992-AU487** **A 19920916**
 PRIORITY INFO.: AU 1991-PK 8367 19910916

TIEN **TNF ANTAGONIST PEPTIDES**

PI **WO 9306128** **A1 19930401**

AI **WO 1992-AU487** **A 19920916**

ABEN The present invention provides **TNF antagonist**
 peptides which have the ability to reduce TNF
 toxicity. The present invention further relates to compositions
 including these peptides as. . .

DETD . . . for the expression of TNF toxicity, TNF
 diminishes the anticoagulant potential of the endothelium,
 inducing procoagulant activity and down regulating the
 expression of **thrombomodulin** (Stern and Nawrothr 1986 J
 Exp Med 163, 740) o
 TNF, a product of activated macrophages produced in
 response to infection and malignancy, was. . .

L10 ANSWER 6 OF 8 USPATFULL on STN

ACCESSION NUMBER: 97:88736 USPATFULL

TITLE: **Tumor necrosis factor antagonists** and their use

INVENTOR(S): Aggarwal, Bharat B., San Mateo, CA, United States
Palladino, Michael A., San Mateo, CA, United States
Shalaby, Mohamed R., San Rafael, CA, United States

PATENT ASSIGNEE(S): Genentech, Inc., South San Francisco, CA, United States
(U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5672347		19970930
APPLICATION INFO.:	US 1995-435934		19950505 (8) <--
RELATED APPLN. INFO.:	Division of Ser. No. US 1994-342676, filed on 21 Nov 1994, now abandoned which is a continuation of Ser. No. US 1993-174212, filed on 28 Dec 1993, now abandoned which is a continuation of Ser. No. US 1993-26717, filed on 5 Mar 1993, now abandoned which is a continuation of Ser. No. US 1991-707412, filed on 28 May 1991, now abandoned which is a continuation of Ser. No. US 1989-417171, filed on 4 Oct 1989, now abandoned which is a continuation of Ser. No. US 1986-898272, filed on 20 Aug 1986, now abandoned which is a continuation-in-part of Ser. No. US 1985-754507, filed on 12 Jul 1985, now abandoned And Ser. No. US 1986-881311, filed on 2 Jul 1986, now abandoned which is a continuation-in-part of Ser. No. US 1984-677156, filed on 3 Dec 1984, now abandoned which is a continuation-in-part of Ser. No. US 1984-627959, filed on 5 Jul 1984, now abandoned		

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Patterson, Jr., Charles L.
LEGAL REPRESENTATIVE: Marschang, Diane L.
NUMBER OF CLAIMS: 7
EXEMPLARY CLAIM: 1
LINE COUNT: 982

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI **Tumor necrosis factor antagonists**
and their use

AI US 1995-435934 19950505 (8) <--

AB **Tumor necrosis factor antagonists**
are administered in therapeutically effective doses to suppress inflammatory immune-potentiated events. The antagonists of this invention typically are selected from among several classes but preferably are neutralizing antibodies directed against **tumor necrosis factor**. The **antagonists** are useful in suppressing transplantation immunity and in the treatment of autoimmune diseases.

SUMM "Lymphotoxin" also was reported to be released by sensitized T lymphocytes from **myocardial infarct** patients exhibiting delayed type hypersensitivity to myoglobin (Mirrakhimov et al., 1985, "Ter Arkh." 56(10): 53-56).

L56 ANSWER 7 OF 26 PCTFULL COPYRIGHT 2003 Univentio on STN
 ACCESSION NUMBER: 1992009203 PCTFULL ED 20020513
 TITLE (ENGLISH): TNF INHIBITORS
 TITLE -(FRENCH): INHIBITEURS DU FACTEUR NECROTIQUE TUMORAL (TNF)
 INVENTOR(S): ESSER, Klaus, Max
 PATENT ASSIGNEE(S): SMITHKLINE BEECHAM CORPORATION;
 ESSER, Klaus, Max
 LANGUAGE OF PUBL.: English
 DOCUMENT TYPE: Patent
 PATENT INFORMATION:

NUMBER	KIND	DATE
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WO 9209203	A1	19920611
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DESIGNATED STATES

W:

AT AU BE CA CH DE DK ES FR GB GR HU IT JP KR LU NL SE
US

APPLICATION INFO.:

WO 1991-US8734	A	19911120
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PRIORITY INFO.:

US 1990-616,479		19901121
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L41 ANSWER 7 OF 37 CAPLUS COPYRIGHT 2003 ACS on STN

ACCESSION NUMBER: 1996:170826 CAPLUS

DOCUMENT NUMBER: 124:220550

TITLE: Treatment for atherosclerosis and other
cardiovascular and inflammatory diseases with
dithiocarboxylates and dithiocarbamates which block
VCAM-1 expression

INVENTOR(S): Medford, Russell M.; Alexander, R. Wayne;
Parthasarathy, Sampath; Khan, Bobby V.; Offermann,
Margaret K.

PATENT ASSIGNEE(S): Emory University, USA

SOURCE: PCT Int. Appl., 99 pp.

CODEN: PIXXD2

DOCUMENT TYPE: Patent

LANGUAGE: English

FAMILY ACC. NUM. COUNT: 3

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 9530415	A1	19951116	WO 1995-US5880	19950510 <--
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RW:	KE, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG			
US 5807884	A	19980915	US 1994-317399	19941004
AU 9525860	A1	19951129	AU 1995-25860	19950510 <--
AU 709939	B2	19990909		
EP 759752	A1	19970305	EP 1995-920396	19950510
R:	AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE			
BR 9507716	A	19970923	BR 1995-7716	19950510
JP 10500111	T2	19980106	JP 1995-529200	19950510
JP 3120091	B2	20001225		
PL 180874	B1	20010430	PL 1995-317193	19950510
NZ 287214	A	20010525	NZ 1995-287214	19950510
PL 184466	B1	20021129	PL 1995-342067	19950510
NO 9604742	A	19961108	NO 1996-4742	19961108 <--
AU 9937951	A1	19990902	AU 1999-37951	19990702
AU 733198	B2	20010510		

PRIORITY APPLN. INFO.:

US 1994-240858	A	19940510
US 1994-317399	A	19941004
US 1992-969934	A2	19921030
AU 1994-56653	A3	19931101
AU 1995-25860	A3	19950510
WO 1995-US5880	W	19950510

OTHER SOURCE(S): MARPAT 124:220550

AB Dithiocarboxylates, including dithiocarbamates, block the induced expression of the endothelial cell surface adhesion mol. VCAM-1, and are therefore useful in the treatment of cardiovascular disease, including atherosclerosis, as well as noncardiovascular inflammatory diseases that are mediated by VCAM-1. Identification of oxidized and unoxidized polyunsatd. fatty acids as direct mediators of VCAM-1 expression is described.

L56 ANSWER 6 OF 26 PCTFULL COPYRIGHT 2003.Univentio on STN
 ACCESSION NUMBER: 1993005780 PCTFULL ED 20020513
 TITLE (ENGLISH): AMINO ACIDS CONTAINING PARENTERAL FORMULATIONS FOR THE
 TREATMENT OF HYPOTENSION AND RELATED PATHOLOGIES
 TITLE (FRENCH): FORMULATIONS CONTENANT DES ACIDES AMINES ADMINISTREES
 PAR VOIE PARENTERALE UTILISEES DANS LE TRAITEMENT DE
 L'HYPOTENSION ET DE PATHOLOGIES APPARENTEES
 INVENTOR(S): KILBOURN, Robert, G.;
 GRIFFITH, Owen, W.;
 GROSS, Steven, S.
 PATENT ASSIGNEE(S): BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM;
 CORNELL RESEARCH FOUNDATION, INC.
 LANGUAGE OF PUBL.: English
 DOCUMENT TYPE: Patent
 PATENT INFORMATION:

NUMBER	KIND	DATE

WO 9305780	A1	19930401

DESIGNATED STATES
 W:

AT AU BB BG BR CA CH CS DE DK ES FI GB HU JP KP MG MN
 MW NL NO PL RO RU SD SE AT BE CH DE DK ES FR GB GR IE
 IT LU MC NL SE BF BJ CF CG CI CM GA GN ML MR SN TD TG

APPLICATION INFO.: WO 1992-US8227 A 19920926
 PRIORITY INFO.: US 1991-767,265 19910927
 US 1992-902,653 19920623
 US 1992-910,868 19920701

L41 ANSWER 13 OF 29 USPATFULL on STN

ACCESSION NUMBER: 97:59188 USPATFULL

TITLE: Low molecular weight sulfated polysaccharides and uses thereof

INVENTOR(S): Shi, Guan Hua, Oingdao, China

PATENT ASSIGNEE(S): Ocean University of Oingdao, Oingdao, China (non-U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5646130		19970708	<--
APPLICATION INFO.:	US 1995-498013		19950630 (8)	
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Kight, John			
ASSISTANT EXAMINER:	Lee, Howard C.			
LEGAL REPRESENTATIVE:	Seidman, StephanieBrown Martin Haller & McClain			
NUMBER OF CLAIMS:	27			
EXEMPLARY CLAIM:	1			
LINE COUNT:	1464			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

PI US 5646130 19970708 <--

DETD The results show that intravenously administered (M.sub.9 G).sub.2 at 6.25, 12.5 and 25 mg/kg had significant anti-**thrombotic** activity. Intravenously administered (M.sub.9 G).sub.2 at 25 mg and 50 mg/kg had significant in vivo **thrombolysis** activity; at 6.25 mg and 25 mg/kg, it had significant ex vivo **thrombolysis** activity. The ED.sub.50 of experimental anti-**thrombotic** activity was 7.2 mg/kg. The anti-**thrombotic** half-life was 55.4 min. In addition, (M.sub.9 G).sub.2 could increase the content of the **serum** fibrin degradative protein (FDP), **decrease serum fibrinogen** (Fg) content and shorten the euglobulin lysis time and increase fibrinolysis activity. The in vivo **thrombolysis** activity is significantly better than anti-**thrombin** enzyme III. At 50 mg/kg, the in vivo **thrombolysis** activity approached 54%, an effect similar to that of urokinase.

L36 ANSWER 27 OF 35 USPATFULL on STN

ACCESSION NUMBER: 83:56129 USPATFULL

TITLE: Diagnostic compositions and method for radiologic imaging of fibrinogen deposition in the body

INVENTOR(S): Wong, Dennis W.; 2853 Sunnyglen Rd., Torrance, CA, United States 90505

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 4418052		19831129	<--
APPLICATION INFO.:	US 1980-177503		19800812	(6)
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Nucker, Christine M.			
NUMBER OF CLAIMS:	15			
EXEMPLARY CLAIM:	1			
LINE COUNT:	411			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A novel injectable diagnostic composition comprises a fibrinolytic enzyme such as streptokinase-activated human plasmin or human urokinase, a stannous reducing agent and an alkaline sodium citrate reagent prepared and packaged as an instant non-radioactive labeling reagent kit to be used in conjunction with a source of .sup.99m Tc-pertechnetate forming a radioactive tracer material suitable for use in scintigraphic imaging of **fibrinogen** or fibrin depositions in **thromboembolic** diseases, in myocardial infarction and in neoplasm.

L36 ANSWER 26 OF 35 USPATFULL on STN

ACCESSION NUMBER: 87:30242 USPATFULL

TITLE: Method of inhibiting and inducing human platelet aggregation

INVENTOR(S): Hawiger, Jack J., Chestnut Hill, MA, United States
Timmons, Sheila, Boston, MA, United States
Lukas, Thomas J., Nashville, TN, United States
Kloczewiak, Marek, Boston, MA, United States

PATENT ASSIGNEE(S): New England Deaconess Hospital, Boston, MA, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 4661471		19870428	<--
APPLICATION INFO.:	US 1984-599477		19840410	(6)
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Phillips, Delbert R.			
LEGAL REPRESENTATIVE:	Lahive & Cockfield			
NUMBER OF CLAIMS:	17			
EXEMPLARY CLAIM:	1			
NUMBER OF DRAWINGS:	1 Drawing Figure(s); 1 Drawing Page(s)			
LINE COUNT:	345			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A method of inhibiting thrombin or ADP-induced human platelet aggregation by **fibrinogen** has been developed. The administration of the small molecular weight peptide or the synthetic inhibitory molecule of the invention significantly inhibits thrombin or ADP-modified human platelets binding of **fibrinogen**, a plasma protein necessary for platelet aggregation. The method of the invention is useful for inhibiting of the formation of hemostatic platelet plugs and of the initiation of **thrombotic** lesions. The blockage caused by hemostatic platelet plugs and the damage caused by **thrombotic** lesions are major factors in heart disease and stroke. The invention also includes a method of inducing the formation of thrombin or ADP-modified platelet aggregates by administration of a synthetic aggregating molecule which represents a functional substitute for **fibrinogen**.

L36 ANSWER 25 OF 35 USPATFULL on STN

ACCESSION NUMBER: 88:80535 USPATFULL

TITLE: Method and compositions for the treatment of thrombotic episodes

INVENTOR(S): Mehta, Jawahar L., Gainesville, FL, United States
Saldeen, Tom G. P., Uppsala, Sweden

PATENT ASSIGNEE(S): University of Florida, Gainesville, FL, United States
(U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 4790988		19881213	<--
APPLICATION INFO.:	US 1987-10603		19870204	(7)
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Phillips, Delbert R.			
ASSISTANT EXAMINER:	Wessendorf, T. D.			
LEGAL REPRESENTATIVE:	Clarke, Dennis P.			
NUMBER OF CLAIMS:	18			
EXEMPLARY CLAIM:	1			
LINE COUNT:	267			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB **Fibrinogen** degradation product pentapeptide 6A and its variants are used in the treatment of thrombi, in particular myocardial infarcts. In a preferred embodiment pentapeptide 6A and tissue plasminogen activator are administered together to enhance the **thrombolytic** activity of tissue plasminogen activator.

ACCESSION NUMBER: 781558 EUROPATFULL EW 199727 FS OS
 TITLE: Pharmaceutical use of heparin cofactor II.
 Pharmazeutische Verwendung von Heparin Kofaktor II.
 Utilisation pharmaceutique du cofacteur II de l'heparine.
 INVENTOR(S): Tsukada, Minoru, c/o The Green Cross Corp., Central Res. Lab., No. 25-1, Shodai Ohtani 22-chome, Hirakata, Osaka 573, JP;
 Matsui, Tomohiko, c/o The Green Cross Corp., No. 3-3, Imabashi 1-chome, Chuo-ku, Osaka 541, JP;
 Shintome, Masakazu, c/o The Green Cross Corp., Central Res. Lab., No. 25-1, Shodai Ohtani 22-chome, Hirakata, Osaka 573, JP;
 Tsuchiyama, Hiromi, c/o The Green Cross Corp. Central Res. Lab., No. 25-1, Shodai Ohtani 2-chome, Hirakata, Osaka 573, JP;
 Maruyama, Tomoyuki, c/o The Green Cross Corp., Central Res. Lab., No. 25-1, Shodai Ohtani 2-chome, Hirakata, Osaka 573, JP
 PATENT ASSIGNEE(S): Tsukada, Minoru, c/o The Green Cross Corp., Central Res. Lab., No. 25-1, Shodai Ohtani 22-chome, Hirakata, Osaka 573, JP;
 Matsui, Tomohiko, c/o The Green Cross Corp., No. 3-3, Imabashi 1-chome, Chuo-ku, Osaka 541, JP;
 Shintome, Masakazu, c/o The Green Cross Corp., Central Res. Lab., No. 25-1, Shodai Ohtani 22-chome, Hirakata, Osaka 573, JP;
 Tsuchiyama, Hiromi, c/o The Green Cross Corp. Central Res. Lab., No. 25-1, Shodai Ohtani 2-chome, Hirakata, Osaka 573, JP;
 Maruyama, Tomoyuki, c/o The Green Cross Corp., Central Res. Lab., No. 25-1, Shodai Ohtani 2-chome, Hirakata, Osaka 573, JP
 PATENT ASSIGNEE NO: 2242600; 2242610; 2242620; 2242630; 2242640
 AGENT: VOSSIUS & PARTNER, Siebertstrasse 4, 81675 Muenchen, DE 100314
 AGENT NUMBER:
 OTHER SOURCE: ESP1997037 EP 0781558 A2 970702
 SOURCE: Wila-EPZ-1997-H27-T1b
 DOCUMENT TYPE: Patent
 LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
 DESIGNATED STATES: R BE; R CH; R DE; R DK; R ES; R FR; R GB; R IT; R LI; R NL; R SE
 PATENT INFO.PUB.TYPE: EPA2 EUROPAEISCHE PATENTANMELDUNG
 PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 781558	A2	19970702

'OFFENLEGUNGS' DATE:	19970702
APPLICATION INFO.:	EP 1996-120816 19961223
PRIORITY APPLN. INFO.:	JP 1995-341789 19951227
PI	EP 781558 A2 19970702

DETDEN. . . acid and arterial blood ketone body ratio], blood platelet count, white blood cell count, clotting factor parameters (fibrinogen, aPTT, PT, TAT and D-dimer) and cytokine (TNF, IL-1 and IL-8). Table 3 shows a part of the results. In all the investigated. . . increase of blood GOT level was observed. It was also suggested that HCII is effective not only for inhibition of **thrombus** formation in an organ but also the **thrombus** formation in vein and artery from the effect of inhibiting **decrease** of **blood fibrinogen**. Moreover, HCII was indicated to be effective for liver function failure and liver deficiency as improvements of blood hyaluronic acid. . .

L41 ANSWER 20 OF 29 USPATFULL on STN

ACCESSION NUMBER: 78:24204 USPATFULL

TITLE: Method of obtaining a splenic composition which inhibits platelet function and said composition

INVENTOR(S): Parmer, Laurence P., 780 River Rd., Lakewood, NJ, United States 08701

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 4088753		19780509	<--
APPLICATION INFO.:	US 1976-688224		19760520 (5)	
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1975-568593, filed on 16 Apr 1975, now abandoned			
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Rosen, Sam			
NUMBER OF CLAIMS:	12			
EXEMPLARY CLAIM:	1,9			
NUMBER OF DRAWINGS:	11 Drawing Figure(s); 8 Drawing Page(s)			
LINE COUNT:	750			

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

PI US 4088753 19780509 <--

SUMM . . . significantly reduce platelet counts, markedly increase bleeding times, reduce or inhibit ADP induced platelet aggregation in vivo and in vitro, **decrease** clottable **plasma** **fibrinogen** concentration, prevent endotoxin produced coagulation, and most importantly prevent experimental **thrombosis**.

SUMM The administration of the novel compounds into dogs disclosed a **decrease** in the quantity of clottable **fibrinogen** in the **plasma**. However, the magnitude of such decrease cannot always be related to the change in platelet count. Decreases of from 20. . . fibrinogen may in part explain the increases in bleeding times, inhibition of ADP induced aggregation and the prevention of experimental **thrombosis**.

L41 ANSWER 15 OF 29 USPATFULL on STN

ACCESSION NUMBER: 96:23094 USPATFULL

TITLE: Method for treating thromboembolic conditions by inhibiting reocclusion via the use of multiple bolus administration of thrombolytically active proteins

INVENTOR(S): Martin, Ulrich, Mannheim, Germany, Federal Republic of Koenig, Reinhard, Grunstadt, Germany, Federal Republic of

PATENT ASSIGNEE(S): Boehringer Mannheim GmbH, Mannheim, Germany, Federal Republic of (non-U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5500411		19960319	<--
	WO 9218157		19921029	<--
APPLICATION INFO.:	US 1994-137116		19940630	(8)
	WO 1992-EP851		19920415	
			19940630	PCT 371 date
			19940630	PCT 102(e) date

	NUMBER	DATE
PRIORITY INFORMATION:	DE 1991-4112398	19910416
	DE 1991-4123845	19910718
DOCUMENT TYPE:	Utility	
FILE SEGMENT:	Granted	
PRIMARY EXAMINER:	Lilling, Herbert J.	
LEGAL REPRESENTATIVE:	Felfe & Lynch	
NUMBER OF CLAIMS:	6	
EXEMPLARY CLAIM:	1	
NUMBER OF DRAWINGS:	5 Drawing Figure(s); 5 Drawing Page(s)	
LINE COUNT:	601	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

PI	US 5500411	19960319	<--
	WO 9218157	19921029	<--

DETD . . . symptoms up to treatment and, also the time from the commencement of treatment up to the dissolving of the coronary **thrombus** is shortened. Thus, more myocardial tissue can be rescued from irreversible destruction. The double or multiple bolus administration according to the invention brings about a higher **thrombolytic** potency of the **thrombolytically** active protein use. In this way, it is possible to reduce dosages as compared to rt-PA. Surprisingly, after bolus injection, . . . longer period of time after the administration. Furthermore, double or multiple bolus injection has the surprising advantage of a smaller **decrease** of **plasma fibrinogen** as compared to single bolus injection. However, these advantageous properties are not achieved with administration of the corresponding total amount of the **thrombolytically** active protein by single bolus injection.

DETD . . . (Table 2). The results shown in Table 3 for the cumulative patency time, the coronary blood flow and the residual **thrombus** weight show that double bolus administration of BM 06.022 significantly increased the coronary blood flow which also remains significantly increased at the end of the experiment, and the residual **thrombus** weight has decreased significantly in comparison with the single bolus injection of 140 KU/kg. FIG. 3 shows that an increase. . . and 140 KU/kg instead of the single bolus injection of the same total dose of BM 06.022 prevents the significant **decrease** of the **plasma fibrinogen** observed previously.

L41 ANSWER 11 OF 29 USPATFULL on STN

ACCESSION NUMBER: 97:109499 USPATFULL

TITLE: Method for treating thromboembolic conditions via the use of multiple bolus administration of thrombolytically active proteins

INVENTOR(S): Martin, Ulrich, Mannheim, Germany, Federal Republic of Konig, Reinhard, San Carlos, CA, United States

PATENT ASSIGNEE(S): Boehringer Mannheim GmbH, Mannheim, Germany, Federal Republic of (non-U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5690931		19971125	<--
APPLICATION INFO.:	US 1994-217616		19940325	(8)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1994-137116, filed on 30 Jun 1994, now patented, Pat. No. US 5500411			

	NUMBER	DATE
PRIORITY INFORMATION:	DE 1991-4112398	19910416
	DE 1991-4123845	19910718
DOCUMENT TYPE:	Utility	
FILE SEGMENT:	Granted	
PRIMARY EXAMINER:	Jacobson, Dian C.	
LEGAL REPRESENTATIVE:	Felfe & Lynch	
NUMBER OF CLAIMS:	20	
EXEMPLARY CLAIM:	1	
NUMBER OF DRAWINGS:	8 Drawing Figure(s); 8 Drawing Page(s)	
LINE COUNT:	877	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

PI US 5690931 19971125 <--

DETD . . . symptoms up to treatment and, also the time from the commencement of treatment up to the dissolving of the coronary **thrombus** is shortened. Thus, more myocardial tissue can be rescued from irreversible destruction. The double or multiple bolus administration according to the invention brings about a higher **thrombolytic** potency of the **thrombolytically** active protein used. In this way, it is possible to reduce dosages as compared to rt-PA. Multiple bolus means that. . . longer period of time after the administration. Furthermore, double or multiple bolus injection has the surprising advantage of a smaller **decrease** of **plasma fibrinogen** as compared to single bolus injection. However, these advantageous properties are not achieved with administration of the corresponding total amount of the **thrombolytically** active protein by single bolus injection.

DETD . . . (Table 2). The results shown in Table 3 for the cumulative patency time, the coronary blood flow and the residual **thrombus** weight show that double bolus administration of BM 06.022 significantly increased the coronary blood flow which also remains significantly increased at the end of the experiment, and the residual **thrombus** weight has decreased significantly in comparison with the single bolus injection of 140 KU/kg. FIG. 3 shows that an increase. . . and 140 KU/kg instead of the single bolus injection of the same total dose of BM 06.022 prevents the significant **decrease** of the **plasma fibrinogen** observed previously.

L7 ANSWER 31 OF 64 MEDLINE DUPLICATE 17

ACCESSION NUMBER: 94297219 MEDLINE

DOCUMENT NUMBER: 94297219 PubMed ID: 8025276

TITLE: Tumor necrosis factor-alpha downregulates protein S secretion in human microvascular and umbilical vein endothelial cells but not in the HepG-2 hepatoma cell line.

AUTHOR: Hooper W C; Phillips D J; Ribeiro M J; Benson J M; George V
G; Ades E W; Evatt B L

CORPORATE SOURCE: Hematologic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333.

SOURCE: BLOOD, (1994 Jul 15) 84 (2) 483-9.
Journal code: A8G; 7603509. ISSN: 0006-4971.

PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals

ENTRY MONTH: 199408

ENTRY DATE: Entered STN: 19940818
Last Updated on STN: 19970203
Entered Medline: 19940811

AB Protein S deficiency, which is associated with **thrombosis**, can either be inherited or acquired. Recently, we reported that a decrease in free protein S was observed in 19 of 25 persons with HIV/AIDS. The proinflammatory cytokine, tumor necrosis factor-alpha (TNF-alpha), has been reported to be elevated in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients and has been shown to induce a procoagulant state on the surface of endothelial cells. We report here that recombinant TNF-alpha (rTNF-alpha) downregulated protein S synthesis in the SV-40T transfected human microvascular endothelial cell line (HMEC-1) model system by approximately 70% and in primary human umbilical vein and dermal microvascular endothelial cell cultures by approximately 50%. Using the HMEC-1 model, Northern blot analysis showed a decrease in protein S RNA at 24 hours that was corroborated by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) quantification. Evidence supporting the specificity of the TNF-alpha effect included the following: (1) TNF-alpha down-regulation of protein S was completely blocked by **TNF** neutralizing **antibody**; (2) the effect was transient, and protein S was restored to near normal levels after TNF was removed from cell cultures; (3) an **antibody** directed to the **TNF** RI (55-kD receptor) was shown to mimic the action of TNF-alpha on HMEC-1 cells; and (4) other proinflammatory cytokines, interleukin (IL)-1, IL-6, and TGF-beta, had no effect on protein S secretion. However, TNF-alpha showed no regulatory control over protein S synthesis in the human hepatocellular carcinoma cell line HepG-2. We suggest that TNF-alpha downregulation of protein S may be a mechanism for localized procoagulant activity and **thrombosis** recently reported in some AIDS patients with associated protein S deficiency.

L7 ANSWER 32 OF 64 MEDLINE DUPLICATE 18

ANSWER 25 OF 26 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1995:280907 BIOSIS
DOCUMENT NUMBER: PREV199598295207
TITLE: The effect of monoclonal **anti-tumor
necrosis factor-antibody** cA2 on
coagulation- and fibrinolytic parameters in
patients with active Crohn's disease.
AUTHOR(S): Hommes, D. W.; Van Dullemen, H. M.; Meenan, J.; Van Den
Ende, A.; Woody, J.; Tytgat, G. N. J.; Van Deventer, S. J.
H.
CORPORATE SOURCE: Cent. Thrombosis, Hemostasis, Atherosclerosis Inflammation
Res., Dep. Gastroenterol., Univ. Amsterdam, Amsterdam
Netherlands
SOURCE: Gastroenterology, (1995) Vol. 108, No. 4 SUPPL., pp.
A838.
Meeting Info.: 95th Annual Meeting of the American
Gastroenterological Association and Digestive Disease Week
San Diego, California, USA May 14-17, 1995
ISSN: 0016-5085.
DOCUMENT TYPE: Conference
LANGUAGE: English

L7 ANSWER 54 OF 64 MEDLINE

ACCESSION NUMBER: 91139354 MEDLINE

DOCUMENT NUMBER: 91139354 PubMed ID: 1995504

TITLE: Role of tumor necrosis factor in the pathogenesis of intravascular coagulopathy of sepsis: potential new therapeutic implications.

AUTHOR: Aderka D

CORPORATE SOURCE: Department of Medicine T, Sourasky Medical Center (Ichilov Hospital), Tel Aviv, Israel.

SOURCE: ISRAEL JOURNAL OF MEDICAL SCIENCES, (1991 Jan) 27 (1) 52-60. Ref: 63
Journal code: GY0; 0013105. ISSN: 0021-2180.

PUB. COUNTRY: Israel
Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199103

ENTRY DATE: Entered STN: 19910412
Last Updated on STN: 19910412
Entered Medline: 19910328

AB Tumor necrosis factor (TNF) induced by bacterial lipopolysaccharide (LPS) was shown to have an important role in precipitation of septic shock and disseminated intravascular clotting (DIC). At the endothelial level TNF down-regulates **thrombomodulin** (thus preventing protein C formation) and inhibits the production of tissue plasminogen activator (t-PA), thus impairing anticoagulant mechanisms. On the other hand, TNF up-regulates the production of procoagulant factors such as t-PA inhibitor (PAI), tissue factor and platelet activating factor (PAF). These effects create an imbalance between procoagulant and anticoagulant mechanisms, in favor of the former. TNF also activates polymorphonuclears (PMNs), and increases their chemotaxis and adherence to endothelial surfaces by up-regulation of specific endothelial (ELAM-1) and PMN (CDw18) adherence proteins. The damage inflicted by activated PMN to the endothelial cell promotes tissue factor exposure and PAI release, with initiation of the characteristic explosive coagulation process of DIC, facilitated by the dissociation between pro- and anticoagulant mechanisms induced by TNF. These newly discovered mechanisms precipitating septic shock and DIC enable consideration of new treatments for this condition as **anti-TNF antibodies** or **TNF inhibitors**, anti-ELAM-1 antibodies anti-tissue factor antibodies, administration of activated factor C, etc. These therapeutic approaches may revolutionize the treatment of septic shock and DIC in the next decade.

L7 ANSWER 55 OF 64 MEDLINE

L7 ANSWER 16 OF 64 MEDLINE

ACCESSION NUMBER: 96097350 MEDLINE

DOCUMENT NUMBER: 96097350 PubMed ID: 8536111

TITLE: Signaling by E-selectin and ICAM-1 induces endothelial tissue factor production via autocrine secretion of platelet-activating factor and tumor necrosis factor

alpha.

AUTHOR: Schmid E; Muller T H; Budzinski R M; Binder K; Pfizenmaier K

CORPORATE SOURCE: Department of Pharmacological Research, Dr. Karl Thomae GmbH, Biberach, Germany.

SOURCE: JOURNAL OF INTERFERON AND CYTOKINE RESEARCH, (1995 Sep) 15 (9) 819-25.

Journal code: CD4; 9507088. ISSN: 1079-9907.

PUB. COUNTRY: United States

Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199602

ENTRY DATE: Entered STN: 19960221

Last Updated on STN: 19970203

Entered Medline: 19960207

AB Based on previous studies showed adhesion molecule-dependent induction of tissue factor upon endothelium-lymphocyte interactions, we investigated whether E-selectin and ICAM-1 are linked to signaling pathways leading to tissue factor gene expression. Cellular interaction was mimicked by antibody cross-linking of E-selectin and ICAM-1 on the surface of human umbilical vein endothelial cells (HUVECs), resulting in induction of tissue factor mRNA and protein expression. Tissue factor production could be independently abolished by **antibodies** against **TNF** -alpha and by WEB 2086, a platelet-activating factor (PAF) receptor antagonist. Because WEB 2086 prevented the production and/or secretion of TNF-alpha by HUVECs, these results provide evidence for E-selectin- and ICAM-1-linked signal pathways leading to tissue factor synthesis in endothelial cells via an autocrine feedback loop involving PAF and TNF-alpha secretion.

L7 ANSWER 17 OF 64 MEDLINE

DUPLICATE 10

L7 ANSWER 24 OF 64 MEDLINE DUPLICATE 13
 ACCESSION NUMBER: 95268957 MEDLINE
 DOCUMENT NUMBER: 95268957 PubMed ID: 7749835
 TITLE: Venous **thrombosis**-associated inflammation and attenuation with neutralizing antibodies to cytokines and adhesion molecules.
 COMMENT: Erratum in: Arterioscler Thromb Vasc Biol 1995 Apr;15(4):550
 AUTHOR: Wakefield T W; Strieter R M; Wilke C A; Kadell A M; Wroblewski S K; Burdick M D; Schmidt R; Kunkel S L; Greenfield L J
 CORPORATE SOURCE: Section of Vascular Surgery, University of Michigan Medical Center, Ann Arbor 48109-0329, USA.
 CONTRACT NUMBER: 1P50HL-46487 (NHLBI)
 AI23521 (NIAID)
 HL50057 (NHLBI)
 SOURCE: ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY, (1995 Feb) 15 (2) 258-68.
 Journal code: B89; 9505803. ISSN: 1079-5642.
 PUB. COUNTRY: United States
 Journal; Article; (JOURNAL ARTICLE)
 LANGUAGE: English
 FILE SEGMENT: Priority Journals
 ENTRY MONTH: 199506
 ENTRY DATE: Entered STN: 19950629
 Last Updated on STN: 19960129
 Entered Medline: 19950621
 AB **Thrombosis** and inflammation are closely related. However, the response of the vein wall to venous **thrombosis** has been poorly documented. This study examines the hypothesis that venous **thrombosis** is associated with an inflammatory response in the vein wall. In a rat model of inferior vena caval **thrombosis**, vein wall was temporally examined for inflammation by assessment of histopathology, leukocyte morphometrics, and cytokine levels. Animals were killed 1 hour and 1, 3, and 6 days after thrombus induction. Our findings demonstrated an early (day 1) neutrophil infiltration into the vein wall followed by a later (days 3 and 6) monocyte/macrophage and lymphocyte response. Cytokines were elevated only under conditions of venous **thrombosis**. Levels of epithelial neutrophil activating protein-78 (ENA-78), tumor necrosis factor-alpha (TNF), interleukin-6, and JE/monocyte chemoattractant protein-1 (JE/MCP-1) increased over the 6-day period, while macrophage inflammatory protein-1 alpha (MIP-1 alpha) peaked at day 3 after thrombus induction. Additionally, rats were passively immunized with neutralizing **antibodies** to **TNF**, ENA-78, MIP-1 alpha, JE/MCP-1, intercellular adhesion molecule-1 (ICAM-1), and CD18 compared with control antibodies. The most effective antibody early after thrombus induction for attenuating vein wall neutrophil extravasation was **anti-TNF** (P < .01). The monocyte/macrophage extravasation was inhibited most by anti-ICAM-1 followed by **anti-TNF** (P < .01). These findings demonstrate that venous **thrombosis** is associated with significant vein wall inflammation that is partially inhibited by neutralizing antibodies to cytokines and adhesion molecules.

L7 ANSWER 28 OF 64 MEDLINE DUPLICATE 15
 ACCESSION NUMBER: 95204555 MEDLINE
 DOCUMENT NUMBER: 95204555 PubMed ID: 7896895
 TITLE: Reciprocal induction of tumor necrosis factor-alpha and interleukin-1 beta activity mediates fibronectin synthesis in **coronary** artery smooth muscle cells.
 AUTHOR: Molossi S; Clausell N; Rabinovitch M
 CORPORATE SOURCE: Division of Cardiovascular Research, Hospital For Sick Children, Toronto, Ontario, Canada.
 SOURCE: JOURNAL OF CELLULAR PHYSIOLOGY, (1995 Apr) 163 (1) 19-29.
 Journal code: HNB; 0050222. ISSN: 0021-9541.
 PUB. COUNTRY: United States
 Journal; Article; (JOURNAL ARTICLE)
 LANGUAGE: English
 FILE SEGMENT: Priority Journals
 ENTRY MONTH: 199504
 ENTRY DATE: Entered STN: 19950504
 Last Updated on STN: 19950504
 Entered Medline: 19950427

AB We previously demonstrated an immune-inflammatory response associated with increased expression of interleukin (IL)-1 beta and fibronectin in graft **coronary** arteriopathy in piglets following heterotopic heart transplant. Further studies showed that increased endogenously produced IL-1 beta was upregulating fibronectin production by donor **coronary** artery (CA) smooth muscle cells (SMC). Since co-induction of IL-1 beta and tumor necrosis factor (TNF)-alpha has been shown in other systems, we investigated the possible interaction between these cytokines in regulating fibronectin production in CA SMC. First, we documented increased TNF-alpha expression in vivo in donor compared to host CA. Next, synthesis of fibronectin was measured in host and donor CA SMC following [35S]-methionine radiolabeling and gelatin-sepharose extraction. As previously shown with IL-1 beta, increased donor CA SMC fibronectin synthesis was reduced to host levels in the presence of **TNF**-alpha **antibodies**, and exogenous **TNF**-alpha upregulated fibronectin synthesis in host CA SMC to levels in donor cells. In normal CA SMC, TNF-alpha-stimulated fibronectin production was downregulated to or below control levels in the presence of IL-1 beta antibodies. Likewise, IL-1 beta-stimulated fibronectin synthesis was downregulated to control levels when **TNF**-alpha neutralizing **antibodies** were added. Combining **TNF**-alpha and IL-1 beta enhanced fibronectin production over that observed with either cytokine alone, but was not additive. Thus, our studies suggest that vascular SMC fibronectin synthesis is regulated by reciprocal induction of IL-1 beta and TNF-alpha activity and provide the first demonstration of a 'cytokine loop' modulating matrix production.

L7 ANSWER 29 OF 64 MEDLINE DUPLICATE 16

L7 ANSWER 55 OF 64 MEDLINE
 ACCESSION NUMBER: 92024820 MEDLINE
 DOCUMENT NUMBER: 92024820 PubMed ID: 1927227
 TITLE: Hypoxic pulmonary vasoconstriction in the adult
 respiratory distress syndrome.
 AUTHOR: Jolin A; Bjertnaes L
 CORPORATE SOURCE: Department of Anesthesiology, University of Tromso,
 Norway.
 SOURCE: ACTA ANAESTHESIOLOGICA SCANDINAVICA. SUPPLEMENTUM,
 (1991) 95 40-52; discussion 53-4. Ref: 113
 Journal code: 08Q; 0370271. ISSN: 0515-2720.
 PUB. COUNTRY: Denmark
 Journal; Article; (JOURNAL ARTICLE)
 General Review; (REVIEW)
 (REVIEW, ACADEMIC)
 LANGUAGE: English
 FILE SEGMENT: Priority Journals
 ENTRY MONTH: 199111
 ENTRY DATE: Entered STN: 19920124
 Last Updated on STN: 19920124
 Entered Medline: 19911107
 AB Increased pulmonary vascular resistance (PVR) and microvascular
 hyperpermeability resulting in lung edema and arterial hypoxemia are
 mainstays in the development of adult respiratory distress syndrome
 (ARDS). The proposed pathophysiologic mechanisms include activation of
 complement and polymorphonuclear leukocytes secreting lysosomal enzymes,
 toxic oxygen metabolites (TOM) and eicosanoids. Platelets and coagulation
 factors are also involved, and in the most severe cases even monocytes
 are
 activated as reflected in release of **thromboplastin**. The latter
 may elicit disseminated intravascular coagulation (DIC). Under
 physiologic
 conditions lung blood flow is diverted from poorly to better oxygenated
 areas by way of hypoxic pulmonary vasoconstriction (HPV), thereby
 counteracting a decrease in arterial oxygenation. Many vasoactive
 substances have been proposed and again refuted as possible mediators of
 HPV. In this study we have focused on the following: histamine,
 catecholamines, arachidonates, calcium, phosphoinositides and TOM as well
 as endothelium-derived relaxing and constricting factors. Whether HPV is
 present in ARDS and whether it is advantageous or not seems to depend on
 the stage and extent of disease. We discuss possible interactions between
 HPV and ARDS mediators and between HPV and various vasoactive agents
 tested for therapeutic effects. Out of the abundance of mediators
 released, prostacyclin, prostaglandin E1, activated complement and
 platelet activating factor have been shown explicitly to inhibit HPV
 whereas others are suspected of doing so. In therapeutical use,
 prostacyclin has proved to reduce PVR and at the same time enhance
 cardiac
 output and oxygen delivery. In mild to moderate ARDS, improvement of
 arterial oxygenation has also been obtained employing almitrine
 bismesylate, a potentiator of HPV. Experimentally, adenosine effectively
 reduces increments in PVR and microvascular permeability with modest
 effects on systemic circulation. However, further investigations are
 warranted to decide whether adenosine or more specific blockers as, for
 instance, monoclonal **antibodies** against **tumor**
necrosis factor should be integrated in ARDS therapy in

the future.

L7 ANSWER 30 OF 64 BIOSIS COPYRIGHT 2001 BIOSIS

ACCESSION NUMBER: 1995:9668 BIOSIS

DOCUMENT NUMBER: PREV199598023968

TITLE: **Anti tumor necrosis factor antibody** inhibited myocardial slippage at non-infarcted myocardium with acute myocardial **infarction**.

AUTHOR(S): Arii, Tohru; Morita, Masato; Iwasaki, Tadaaki

CORPORATE SOURCE: Hyogo College Med., Nishinomiya Japan

SOURCE: Circulation, (1994) Vol. 90, No. 4 PART 2, pp. I522.

Meeting Info.: 67th Scientific Sessions of the American Heart Association Dallas, Texas, USA November 14-17, 1994
ISSN: 0009-7322.

DOCUMENT TYPE: Conference

LANGUAGE: English

L7 ANSWER 39 OF 64 MEDLINE DUPLICATE 23

ACCESSION NUMBER: 93374001 MEDLINE

DOCUMENT NUMBER: 93374001 PubMed ID: 8396037

TITLE: Tumor necrosis factor involvement in myocardial ischaemia-reperfusion injury.

AUTHOR: Squadrito F; Altavilla D; Zingarelli B; Ioculano M; Calapai

CORPORATE SOURCE: G; Campo G M; Miceli A; Caputi A P
Institute of Pharmacology, School of Medicine, University of Messina, Italy.

SOURCE: EUROPEAN JOURNAL OF PHARMACOLOGY, (1993 Jun 24)
237 (2-3) 223-30.
Journal code: EN6; 1254354. ISSN: 0014-2999.

PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199310

ENTRY DATE: Entered STN: 19931022
Last Updated on STN: 19970203
Entered Medline: 19931004

AB The role of tumor necrosis factor-alpha was investigated in an anaesthetized rat model of **coronary** artery ligation (60 min) and reperfusion (MI/R). Sham-occluded rats (sham MI/R) were used as controls. Survival rate, myocardial necrosis, myocardial myeloperoxidase activity, serum creatinine kinase activity and serum and macrophage tumor necrosis factor-alpha were studied. Ischaemia-reperfusion injury significantly reduced survival rate (45%), produced marked myocardial injury, increased serum creatinine kinase activity and increased myocardial myeloperoxidase activity in the area-at-risk and in the necrotic area. Serum tumor necrosis factor-alpha was undetectable during the occlusion period, but increased significantly upon release of the **coronary** artery. At the end of reperfusion, macrophage tumor necrosis factor-alpha was also increased. Passive immunization with a hyperimmune serum containing **antibodies** against murine **tumor necrosis factor-alpha** significantly increased survival rate (80%), lowered myocardial necrosis, reduced the increase in serum creatinine kinase activity and decreased myeloperoxidase activity in the area-at-risk and in the necrotic area. These data are consistent with an involvement of tumor necrosis factor-alpha in myocardial ischaemia-reperfusion injury.

L7 ANSWER 40 OF 64 MEDLINE

not actually
thrombosis

L7 ANSWER 43 OF 64 MEDLINE

ACCESSION NUMBER: 93272254 MEDLINE

DOCUMENT NUMBER: 93272254 PubMed ID: 8388776

TITLE: [Tumor necrosis factor in myocardial ischemia and reperfusion].
Tumor necrosis factor nell'ischemia e riperfusione miocardica.

AUTHOR: Squadrito F; Saitta A; Altavilla D; Campo G M; Ioculano M; Squadrito G; Caputi A P

CORPORATE SOURCE: Istituto di Farmacologia, Universita degli Studi, Messina.

SOURCE: CARDIOLOGIA, (1993 Jan) 38 (1) 45-51.
Journal code: COE; 8506637. ISSN: 0393-1978.

PUB. COUNTRY: Italy
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: Italian

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199306

ENTRY DATE: Entered STN: 19930716

Last Updated on STN: 19930716

Entered Medline: 19930628

AB The role of tumor necrosis factor (TNF-alpha) was investigated in an anaesthetized rat model of **coronary** artery ligation (60 min) followed by reperfusion (60 min; MI/R). Sham operated rats were used as controls (Sham MI/R). Myocardial necrosis, myocardial myeloperoxidase activity (MPO; investigated as an index of leukocyte adhesion and accumulation), serum creatinphosphokinase (CPK) activity and serum and macrophage TNF-alpha were studied. Ischemia and reperfusion produced a marked myocardial injury, with enhancement of serum CPK levels and myocardial MPO activity in the area at risk and in the necrotic area. Furthermore, serum TNF-alpha was undetectable during the occlusion period, but increased significantly after release of the **coronary** artery. At the end of reperfusion, macrophage TNF-alpha was also enhanced.

A passive immunization with a hyperimmune serum containing **antibodies** against murine **TNF-alpha** or administration of an inhibitor of TNF-alpha synthesis, such as cloricromene, significantly lowered myocardial necrosis, reduced the increase in serum CPK and decreased MPO activity in the area at risk and in the necrotic area. Finally, the administration of the specific **anti-TNF-alpha antibodies** neutralized the serum levels of TNF-alpha and the injection of cloricromene reduced both serum and macrophage TNF-alpha.

These data are consistent with an involvement of TNF-alpha in myocardial ischemia-reperfusion injury and suggest that drugs capable of reducing TNF-alpha might represent a novel therapeutic approach to the treatment of myocardial reperfusion injury.

L7 ANSWER 44 OF 64 MEDLINE

L7 ANSWER 38 OF 64 MEDLINE

DUPLICATE 22

ACCESSION NUMBER: 93233092 MEDLINE
DOCUMENT NUMBER: 93233092 PubMed ID: 8097251
TITLE: In vitro correlates of the L. casei animal model of
Kawasaki disease.
AUTHOR: Tomita S; Myones B L; Shulman S T
CORPORATE SOURCE: Division of Infectious Diseases, Children's Memorial
Hospital, Chicago, IL 60614.
SOURCE: JOURNAL OF RHEUMATOLOGY, (1993 Feb) 20 (2) 362-7.
Journal code: JWX; 7501984. ISSN: 0315-162X.
PUB. COUNTRY: Canada
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199305
ENTRY DATE: Entered STN: 19930604
Last Updated on STN: 19950206
Entered Medline: 19930518

AB The induction of **coronary** arteritis in mice by Lactobacillus
casei cell wall (CW) is thought to represent an animal model of Kawasaki
disease. Treatment of vascular endothelial cells (EC) in vitro with
supernatants from CW stimulated human mononuclear cells (MNC) enhanced
adherence of human polymorphonuclear leukocyte (PMN) to human EC, and EC
expression of intercellular adhesion molecule-1 (ICAM-1) but not HLA-DR.
Supernatants contained high concentrations of tumor necrosis factor-alpha
(TNF-alpha) and PMN adherence correlated directly with the concentration
of TNF-alpha. Intravenous human gamma globulin (IVGG) preparations did
not
block the effect of cytokine containing MNC supernates upon EC, ICAM-1
expression by EC, or PMN adherence to prestimulated EC. However, both EC
ICAM-1 expression and enhanced PMN adherence to EC by CW induced MNC
supernatants were blocked by **anti-TNF**-alpha treatment.
The initial **coronary** inflammatory reaction in the mouse model
appears to involve PMN adherence to vascular EC that have been activated
by TNF-alpha released by MNC after stimulation with CW.

L7 ANSWER 39 OF 64 MEDLINE

DUPLICATE 23

L6 ANSWER 98 OF 99 BIOSIS COPYRIGHT 2001 BIOSIS
ACCESSION NUMBER: 1989:278991 BIOSIS
DOCUMENT NUMBER: BR37:3988
TITLE: SUPPRESSION OF LACTOBACILLUS-CASEI CELL WALL-INDUCED
CORONARY ARTERITIS IN MICE BY RABBIT
ANTIBODY TO MURINE **TUMOR NECROSIS**
FACTOR TNF.
AUTHOR(S): LEHMAN T J A; GIETL D; NGUYEN H T; SHERRY B; CERAMI A
CORPORATE SOURCE: CORNELL UNIV. MED. CENT., HOSP. SPECIAL SURGERY, PEDIATRIC
RHEUMATOL., NEW YORK, N.Y. 10021.
SOURCE: JOINT MEETING OF THE AMERICAN PEDIATRIC SOCIETY AND THE
SOCIETY FOR PEDIATRIC RESEARCH, WASHINGTON, D.C., USA, MAY
1-4, 1989. PEDIATR RES, (1989) 25 (4 PART 2), 27A.
CODEN: PEREBL. ISSN: 0031-3998.
DOCUMENT TYPE: Conference
FILE SEGMENT: BR; OLD
LANGUAGE: English

pentoxifylline

PATENT APPLICATION - PATENTANMELDUNG - DEMANDE DE BREVET

ACCESSION NUMBER: 411754 EUROPATFULL EW 199106 FS OS STA B
 TITLE: Medicament for the inhibition of interleukin-1 or tumor necrosis factor production by monocytes and/or macrophages.
 Medikament zur Inhibierung der Interleukin-1- oder Tumornekrosefaktorproduktion durch Monocyten und/oder Makrophagen.
 Medicament pour l'inhibition de la production de l'interleucine-1 ou du facteur de necrose tumorale par des monocytes et/ou macrophages.
 INVENTOR(S): Badger, Alison Mary, 56 Parkridge Drive, Bryn Mawr, Pennsylvania 19010, US;
 Esser, Klaus Max, 200 Hickory Drive, Downingtown, Pennsylvania 19335, US;
 Bender, Paul Elliot, 504 Lilac Lane, Cherry Hill, New Jersey 08003, US;
 Griswold, Don Edgar, 205 Lower Valley Road, North Wales, Pennsylvania 19454, US;
 Hanna, Nabil, 11099 North Torrey Pines Road, La Jolla, California 92037, US;
 Votta, Bartholomew Jude, 1465 North Valley Road, Pottstown, Pennsylvania 19464, US;
 Lee, John C., 245 Gulph Hills Road, Radnor, Pennsylvania 19087, US;
 Simon, Philip Leonard, 18 Leigh Court, Randolph, New Jersey 07869, US
 PATENT ASSIGNEE(S): SMITHKLINE BEECHAM CORPORATION, P.O. Box 7929 1 Franklin Plaza, Philadelphia Pennsylvania 19101, US
 PATENT ASSIGNEE NO: 201243
 AGENT: Giddings, Peter John, Dr. et al, SmithKline Beecham, Corporate Patents, Mundells, Welwyn Garden City, Hertfordshire AL7 1EY, GB
 AGENT NUMBER: 55332
 OTHER SOURCE: ESP1991011 EP 0411754 A2 910206
 SOURCE: Wila-EPZ-1991-H06-T1
 DOCUMENT TYPE: Patent
 LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
 DESIGNATED STATES: R AT; R BE; R CH; R DE; R DK; R ES; R FR; R GB; R GR; R IT; R LI; R LU; R NL; R SE
 PATENT INFO.PUB.TYPE: EPA2 EUROPAEISCHE PATENTANMELDUNG
 PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 411754	A2	19910206
EP 1990-306437		19900613
US 1989-365349		19890613
AI EP 1990-306437		19900613
PI EP 411754	A2	19910206

'OFFENLEGUNGS' DATE: 19910206
 APPLICATION INFO.: EP 1990-306437 19900613
 PRIORITY APPLN. INFO.: US 1989-365349 19890613
 AI EP 1990-306437 19900613
 PI EP 411754 A2 19910206
 DETDEN. . . . experimental cerebral malaria (ECM) that reproduces some features of the human disease was prevented in mice by administration of an anti-TNF antibody [See, Grau et al., Imm. Review 112:49-70 (1989)]. Levels of serum TNF correlated directly with the severity of disease and. . . . Disease. The deposition of silica particles leads to silicosis, a disease of progressive respiratory failure caused by a fibrotic reaction. Antibody to TNF completely blocked the silica-induced lung fibrosis in mice [See Piguet et al., Nature, 344:245-247 (1990)]. High levels of TNF production. . . . blood flow. [See, Vedder et al., PNAS 87:2643-2646 (1990)]; TNF also alters the properties of endothelial cells and has various pro-coagulant activities, such as producing an increase in tissue factor pro.shy. coagulant activity and

suppression of the **anticoagulant** protein C pathway as well as down-regulating the expression of **thrombomodulin** [See, Sherry et al., J.Cell Biol. 107:11269-1277 (1988)]. TNF also has pro-inflammatory activities which together with its early production (during . . . an inflammatory event)—make it a ~~likely mediator of~~ tissue injury in several important disorders including but not limited to, **myocardial infarction**, stroke and circulatory shock. Of specific importance may be TNF-induced expression of adhesion molecules, such as intercellular adhesion molecule (ICAM). . .

L5 ANSWER 2 OF 11 BIOTECHDS COPYRIGHT 2003 THOMSON DERWENT/ISI on STN

ACCESSION NUMBER: 2003-14054 BIOTECHDS

TITLE: Novel **antibody** which binds to human **tumor necrosis factor** related apoptosis inducing ligand protein, useful for inhibiting TRAIL-mediated apoptosis of a target cell, or blocking binding of TRAIL to a target cell;
mouse hybridoma cell culture for monoclonal antibody production for use in disease therapy

AUTHOR: WILEY S R; GOODWIN R G

PATENT ASSIGNEE: IMMUNEX CORP

PATENT INFO: US 6521228 18 Feb 2003

APPLICATION INFO: US 2001-825563 2 Apr 2001

PRIORITY INFO: US 2001-825563 2 Apr 2001; US 1995-496632 29 Jun 1995

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: WPI: 2003-340628 [32]

AN 2003-14054 BIOTECHDS

AB DERWENT ABSTRACT:

NOVELTY - An antibody (I) that specifically binds: (a) the human tumor necrosis factor related apoptosis inducing ligand (TRAIL) protein having a fully defined sequence of 281 amino acids (S2) as given in the specification; (b) a soluble human TRAIL polypeptide; (c) a polypeptide comprising amino acids 124-276 of (S2), or (d) a fragment of the TRAIL protein of (S2), is new.

DETAILED DESCRIPTION - An antibody (I) that specifically binds: (a) the human tumor necrosis factor related apoptosis inducing ligand (TRAIL) protein having a fully defined sequence of 281 amino acids (S2) as given in the specification; (b) a soluble human TRAIL polypeptide, where the polypeptide comprises amino acids x to 281 of (S2), where x represents an integer from 39 to 95; (c) a polypeptide comprising amino acids 124-276 of (S2); or (d) a fragment of the TRAIL protein of (S2), where the N-terminal amino acid of the fragment is selected from residues 39-124 of (S2), and the C-terminal amino acid of the fragment is selected from residues 276-281 of (S2). INDEPENDENT CLAIMS are also included for the following: (1) an antigen-binding fragment of (I) (a monoclonal antibody); (2) hybridoma cell line that produces (I) which is a monoclonal antibody that specifically binds human TRAIL protein having a sequence of (S2), a soluble fragment of (S2), or a fragment of (S2); and (3) a composition comprising (I) (a monoclonal antibody) that specifically binds human TRAIL protein having a sequence of (S2), and a carrier, diluent, or excipient.

BIOTECHNOLOGY - Preferred Antibody: (I) is a monoclonal antibody, and inhibits TRAIL-mediated apoptosis of a target cell, or blocks binding of TRAIL to a target cell.

ACTIVITY - Hemostatic; Immunosuppressive; Antiinflammatory; Dermatological.

MECHANISM OF ACTION - Inhibits TRAIL-mediated apoptosis of a target cell; Blocks binding of TRAIL to a target cell (claimed). Human microvascular endothelial cells of dermal origin were treated for 16-18 hours with plasma from patients with thrombotic thrombocytopenic purpura (TTP) or with control plasma, either alone or in the presence of anti-TRAIL polyclonal antiserum. A 1:2000 dilution of the antiserum was employed. The plasma was from two TTP patients, designated 1 and 2. The cells employed in the assays were MVEC-1 and MVEC-2. Results showed that plasma derived from TTP patients induced apoptosis of microvascular endothelial cells of dermal origin. This apoptosis was inhibited by polyclonal antibodies directed against TRAIL.

USE - (I) is used in assays to detect the presence of TRAIL polypeptides, either in vitro or in vivo, purifying TRAIL by affinity chromatography, blocking binding of TRAIL to target cells and thus inhibiting a biological activity of TRAIL. (I) is useful for treating disorders mediated or exacerbated by TRAIL. For example (I) is useful for

treating thrombotic microangiopathies, e.g., thrombotic thrombocytopenic purpura (TTP). (I) is also useful for treating adult hemolytic uremic syndrome (HUS) (even though it can strike children as well, and for treating conditions mediated by clotting of small blood vessels e.g., cardiac problems in pediatric AIDS patients. (I) is also useful for treating systemic lupus erythematosus (SLE).

EXAMPLE - Mice were immunized with tumor necrosis factor related apoptosis inducing ligand (TRAIL) as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 microg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals were boosted with additional TRAIL emulsified in incomplete Freund's adjuvant. Mice were periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples were periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot blot assay or enzyme-Linked immunosorbent assay (ELISA) for TRAIL antibodies. Following detection of an appropriate antibody titer, positive animals were provided one last intravenous injection of TRAIL in saline. Three to four days later, the animals were sacrificed, spleen cells harvested, and spleen cells were fused to a murine myeloma cell line such as NSS1 or, preferably, P3x63Ag 8.653 (ATTC CRL 1580). Fusions generate hybridoma cells, which were plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids. The hybridoma cells were screened by ELISA for reactivity against purified TRAIL. Positive hybridoma cells were injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL monoclonal antibodies. (40 pages)

L11 ANSWER 2 OF 4 MEDLINE
 ACCESSION NUMBER: 95393687 MEDLINE
 DOCUMENT NUMBER: 95393687 PubMed ID: 7664546
 TITLE: CDP571, a humanized antibody to human tumor necrosis factor-alpha: safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock. CPD571 Sepsis Study Group.
 AUTHOR: Dhainaut J F; Vincent J L; Richard C; Lejeune P; Martin C; Fierobe L; Stephens S; Ney U M; Sopwith M
 CORPORATE SOURCE: Intensive Care Units, Cochin Port-Royal University Hospital, Paris, France.
 SOURCE: CRITICAL CARE MEDICINE, (1995 Sep) 23 (9) 1461-9. Journal code: DTF; 0355501. ISSN: 0090-3493.
 PUB. COUNTRY: United States
 (CLINICAL TRIAL)
 (CLINICAL TRIAL, PHASE II)
 Journal; Article; (JOURNAL ARTICLE)
 (MULTICENTER STUDY)
 (RANDOMIZED CONTROLLED TRIAL)
 LANGUAGE: English
 FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
 ENTRY MONTH: 199510
 ENTRY DATE: Entered STN: 19951020
 Last Updated on STN: 19951020
 Entered Medline: 19951011
 AB OBJECTIVES: To determine the safety of a "humanized" antibody to human **anti-tumor necrosis factor** -alpha (TNF-alpha) in patients with septic shock, and to examine the pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in this patient group. DESIGN: Prospective, randomized, placebo-controlled, phase II multicenter clinical trial, with escalating doses of a fully **humanized anti-TNF** -alpha antibody (CDP571). SETTING: Seven academic intensive care units in Europe. PATIENTS: Forty-two patients with rapidly evolving septic shock who received CDP571 in addition to standard supportive care. INTERVENTIONS: Patients received intravenously either placebo or one of four single doses of CDP571: 0.1, 0.3, 1.0, or 3.0 mg/kg. MEASUREMENTS AND MAIN RESULTS: The **humanized anti-TNF-alpha** antibody was well tolerated. The overall all-cause 28-day mortality rate was 62%. Mortality rate was similar in the placebo and treatment groups, except that all six patients who received 0.3 mg/kg of CDP571 died within 7 days. This outcome, which was not dose-related, is consistent with the poorer prognostic characteristics of this group at baseline. The peak CDP571 concentrations and area under the curve increased proportionately with the dose. The low level of the immune response detected had little effect on the ability of circulating CDP571 to bind TNF-alpha and on the pharmacokinetics of the antibody. An abrupt reduction in circulating TNF-alpha concentration was observed 30 mins after CDP571 administration at all active dosage levels. While interleukin-1 beta and interleukin-6 plasma concentrations decreased with time in all dosage groups, these cytokine concentrations decreased more rapidly during the initial 24 hrs in the treatment groups than in the placebo group. CONCLUSIONS: The **humanized anti-TNF-alpha** antibody, CDP571, is well tolerated and able to cause a dose-dependent reduction in circulating

TNF-alpha concentrations in patients with septic shock. Further studies are needed to determine the efficacy of this antibody to improve the survival rates of critically ill patients with severe sepsis.

L11 ANSWER 3 OF 4 MEDLINE
ACCESSION NUMBER: 95012256 MEDLINE
DOCUMENT NUMBER: 95012256 PubMed ID: 7927362
TITLE: A humanized anti-tumor
necrosis factor-alpha monoclonal antibody
that acts as a partial, competitive antagonist of the
template antibody.
AUTHOR: Tempest P R; Barbanti E; Bremner P; Carr F J; Ghislieri M;
Rifaldi B; Marcucci F
CORPORATE SOURCE: Scotgen Biopharmaceuticals Inc., Aberdeen, Scotland, UK.
SOURCE: HYBRIDOMA, (1994 Jun) 13 (3) 183-90.
Journal code: GFS; 8202424. ISSN: 0272-457X.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-Z22669; GENBANK-Z22670
ENTRY MONTH: 199411
ENTRY DATE: Entered STN: 19941222
Last Updated on STN: 19941222
Entered Medline: 19941123

AB We have constructed several humanized versions of a monoclonal antibody (MAb78) against human tumor necrosis factor-alpha (huTNF-alpha) retaining the complementarity-determining regions (CDR) of the original mouse MAb with or without a variable number of original framework region (FR) residues. All versions, except one, showed a loss of binding affinity and neutralizing potency of at least 10-fold compared to the original mouse MAb or its chimeric equivalent. In some cases, however, the decrease in neutralizing potency was significantly greater than the decrease in binding affinity. Two humanized versions showing the greatest

dissociation

between these two parameters were studied for their capacity to inhibit the neutralizing activity of chimeric or murine MAb78 when used at concentrations that bound but only partially neutralized huTNF-alpha. One humanized version (MAb78D) was indeed able to do so, whereas the other (MAb78C) was not found to exert any inhibitory activity at all concentrations tested. The antagonistic effect of MAb78D was

concentration

dependent and could be overcome by increasing the concentrations of chimeric or murine MAb78. Two different models of MAb78-huTNF-alpha interaction that may help explain the antagonist activity of humanized MAb78D are discussed.

L11 ANSWER 4 OF 4 MEDLINE
ACCESSION NUMBER: 94118289 MEDLINE
DOCUMENT NUMBER: 94118289 PubMed ID: 8289265
TITLE: Idiotope determining regions of a mouse monoclonal
antibody
and its humanized versions. Identification of framework
residues that affect idiotype expression.
AUTHOR: Corti A; Barbanti E; Tempest P R; Carr F J; Marcucci F
CORPORATE SOURCE: Molecular Immunology and Biochemistry Unit, Tecnogen SCpA,
Milan, Italy.
SOURCE: JOURNAL OF MOLECULAR BIOLOGY, (1994 Jan 7) 235

(1) 53-60.

Journal code: J6V; 2985088R. ISSN: 0022-2836.

PUB. COUNTRY:

ENGLAND: United Kingdom

Journal; Article; (JOURNAL ARTICLE)

LANGUAGE:

English

FILE SEGMENT:

Priority Journals

ENTRY MONTH:

199402

ENTRY DATE:

Entered STN: 19940312

Last Updated on STN: 19940312

Entered Medline: 19940224

AB The contribution of framework regions (FRs) of antibody-variable domains to idiotype expression was studied by examining the interaction of various

"humanized" versions of a mouse anti-TNF

alpha monoclonal antibody (mAb78) with polyclonal and two monoclonal antibodies (mAb1G3 and mAb9F1), generated against the mAb78 idiotype.

Humanized mAb78, bearing human constant domains and mouse

complementarity-determining regions (CDRs) inserted with human FRs, was

found to be five to sevenfold less reactive than mAb78 with polyclonal

anti-idiotype antibodies and 200 to 300-fold less active in neutralizing

TNF alpha. The substitution of heavy-chain FRs residues of the humanized

antibody with original mouse residues 28 to 30, 48 to 49, 67 to 68, 70 to

71, 78, 80 and 82 progressively restored the immunoreactivity with

polyclonal immunoglobulin Gs to the level of a version having mouse heavy

chain and human light chain FRs, and increased 10 to 20-fold the TNF

alpha

neutralizing activity. This suggests that at least some of these residues

are critical for TNF alpha binding as well as for the expression of

idiotopes that are strongly immunogenic in syngeneic animals. All

antibody

versions with either human or mouse FRs were able to bind to various

extents mAb1G3, a gamma-type anti-Id antibody that inhibits mAb78/TNF

alpha interaction by paratope blockade. At variance, only the antibody

versions containing mouse FRs were able to bind mAb9F1, an alpha-type

anti-Id antibody unable to block the access of TNF alpha to mAb78

paratopes. Substitution of heavy chain FR residues 28 to 30 markedly

decreased the binding of mAb1G3 (100 to 1000-fold). This suggests that

these antibodies recognize CDR and FR idiotopes, respectively, that can

be

drastically modified by changes in the FRs. In conclusion, the results

suggest that CDRs as well as FRs markedly contribute to antibody Id

expression. Although strongly immunogenic idiotopes are probably located

within the CDRs, the results also suggest that some FR residues are

critically involved in shaping antibody Id diversity by affecting the

structure of CDR-related idiotopes.

✓

L11 ANSWER 2 OF 4 MEDLINE

ACCESSION NUMBER: 95393687 MEDLINE

DOCUMENT NUMBER: 95393687 PubMed ID: 7664546

TITLE: CDP571, a humanized antibody to human tumor necrosis factor-alpha: safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock. CPD571 Sepsis Study Group.

AUTHOR: Dhainaut J F; Vincent J L; Richard C; Lejeune P; Martin C; Fierobe L; Stephens S; Ney U M; Sopwith M

CORPORATE SOURCE: Intensive Care Units, Cochin Port-Royal University Hospital, Paris, France.

SOURCE: CRITICAL CARE MEDICINE, (1995 Sep) 23 (9) 1461-9. Journal code: DTF; 0355501. ISSN: 0090-3493.

PUB. COUNTRY: United States
(CLINICAL TRIAL)
(CLINICAL TRIAL, PHASE II)
Journal; Article; (JOURNAL ARTICLE)
(MULTICENTER STUDY)
(RANDOMIZED CONTROLLED TRIAL)

LANGUAGE: English

FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals

ENTRY MONTH: 199510

ENTRY DATE: Entered STN: 19951020
Last Updated on STN: 19951020
Entered Medline: 19951011

AB OBJECTIVES: To determine the safety of a "humanized" antibody to human **anti-tumor necrosis factor** -alpha (TNF-alpha) in patients with septic shock, and to examine the pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in this patient group. DESIGN: Prospective, randomized, placebo-controlled, phase II multicenter clinical trial, with escalating doses of a fully **humanized anti-TNF** -alpha antibody (CDP571). SETTING: Seven academic intensive care units in Europe. PATIENTS: Forty-two patients with rapidly evolving septic shock who received CDP571 in addition to standard supportive care. INTERVENTIONS: Patients received intravenously either placebo or one of four single doses of CDP571: 0.1, 0.3, 1.0, or 3.0 mg/kg. MEASUREMENTS AND

MAIN RESULTS: The **humanized anti-TNF-alpha** antibody was well tolerated. The overall all-cause 28-day mortality rate was 62%. Mortality rate was similar in the placebo and treatment groups, except that all six patients who received 0.3 mg/kg of CDP571 died within 7 days. This outcome, which was not dose-related, is consistent with the poorer prognostic characteristics of this group at baseline. The peak CDP571 concentrations and area under the curve increased proportionately with the dose. The low level of the immune response detected had little effect on the ability of circulating CDP571 to bind TNF-alpha and on the pharmacokinetics of the antibody. An abrupt reduction in circulating TNF-alpha concentration was observed 30 mins after CDP571 administration at all active dosage levels. While interleukin-1 beta and interleukin-6 plasma concentrations decreased with time in all dosage groups, these cytokine concentrations decreased more rapidly during the initial 24 hrs in the treatment groups than in the placebo group. CONCLUSIONS: The **humanized anti-TNF-alpha** antibody, CDP571, is well tolerated and able to cause a dose-dependent reduction in circulating

TNF-alpha concentrations in patients with septic shock. Further studies are needed to determine the efficacy of this antibody to improve the survival rates of critically ill patients with severe sepsis.

L11 ANSWER 3 OF 4 MEDLINE

W
 L8 ANSWER 1 OF 1 MEDLINE
 ACCESSION NUMBER: 93178084 MEDLINE
 DOCUMENT NUMBER: 93178084 PubMed ID: 8440099
 TITLE: Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. The CB0006 Sepsis Syndrome Study Group.
 COMMENT: Comment in: Crit Care Med. 1993 Mar;21(3):311-2
 Comment in: Crit Care Med. 1994 Oct;22(10):1702-3
 AUTHOR: Fisher C J Jr; Opal S M; Dhainaut J F; Stephens S; Zimmerman J L; Nightingale P; Harris S J; Schein R M; Panacek E A; Vincent J L; +
 CORPORATE SOURCE: Department of Pulmonary, Cleveland Clinic Foundation 44195.
 SOURCE: CRITICAL CARE MEDICINE, (1993 Mar) 21 (3) 318-27.
 Journal code: DTF; 0355501. ISSN: 0090-3493.
 PUB. COUNTRY: United States
 (CLINICAL TRIAL)
 (CLINICAL TRIAL, PHASE II)
 (CONTROLLED CLINICAL TRIAL)
 Journal; Article; (JOURNAL ARTICLE)
 (MULTICENTER STUDY)
 LANGUAGE: English
 FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
 ENTRY MONTH: 199303
 ENTRY DATE: Entered STN: 19930416
 Last Updated on STN: 19960129
 Entered Medline: 19930331
 AB OBJECTIVES: To determine the safety, pharmacokinetics, and activity of an anti-tumor necrosis factor (TNF)-alpha monoclonal antibody in severe sepsis. DESIGN: Open-label, prospective, phase II multicenter trial with escalating doses of a murine monoclonal antibody (CB0006). SETTING: Twelve academic medical center intensive care units in the United States and Europe. PATIENTS: Eighty patients with severe sepsis or septic shock who received standard supportive care and antimicrobial therapy in addition to the anti-TNF antibody. INTERVENTIONS: Patients were treated intravenously with one of four dosing regimens with CB0006: 0.1 mg/kg, 1.0 mg/kg, 10 mg/kg or two doses of 1 mg/kg 24 hrs apart. MEASUREMENTS AND MAIN RESULTS: The murine monoclonal anti-TNF antibody was well tolerated despite the development of anti-murine antibodies in 98% of patients. No survival benefit was found for the total study population, but patients with increased circulating TNF concentrations at study entry appeared to benefit by the high dose anti-TNF antibody treatment. Increased interleukin (IL)-6 levels predicted a fatal outcome ($p = .003$), but TNF levels were not found to be a prognostic indicator. TNF levels were higher (206.7 +/- 60.7 vs. 85.9 +/- 26.1 pg/mL; $p < .001$) and outcome was poor (41% vs. 71% survival; $p = .007$) in patients who were in shock at study entry when compared with septic patients not in shock. CONCLUSIONS: The murine anti-TNF-alpha monoclonal antibody CB0006 has proven to be safe in this clinical trial and may prove to be useful in septic patients with increased circulating TNF concentrations. Further studies are needed to determine efficacy and the ultimate clinical utility of this immunotherapeutic agent in sepsis.

L25 ANSWER 55 OF 58 SCISEARCH COPYRIGHT 2003 THOMSON ISI on STN
 ACCESSION NUMBER: 91:508763 SCISEARCH
 THE GENUINE ARTICLE: GE530
 TITLE: CORONARY ATHEROSCLEROTIC PLAQUES WITH AND WITHOUT
THROMBUS IN ISCHEMIC **HEART** SYNDROMES - A
 MORPHOLOGICAL, IMMUNOHISTOCHEMICAL, AND BIOCHEMICAL-STUDY
 AUTHOR: ARBUSTINI E (Reprint); GRASSO M; DIEGOLI M; PUCCI A;
 BRAMERIO M; ARDISSINO D; ANGOLI L; DESERVI S; BRAMUCCI E;
 MUSSINI A; MINZIONI G; VIGANO M; SPECCHIA G
 CORPORATE SOURCE: UNIV PAVIA, POLICLIN SAN MATTEO, IST RIC & CURA CARATTERE
 SCI, DEPT PATHOL, I-27100 PAVIA, ITALY; UNIV PAVIA,
 POLICLIN SAN MATTEO, IST RIC & CURA CARATTERE SCI, DEPT
 CARDIOL, I-27100 PAVIA, ITALY; UNIV PAVIA, POLICLIN SAN
 MATTEO, IST RIC & CURA CARATTERE SCI, DEPT CARDIAC SURG,
 I-27100 PAVIA, ITALY
 COUNTRY OF AUTHOR: ITALY
 SOURCE: AMERICAN JOURNAL OF CARDIOLOGY, (1991) Vol. 68,
 No. 7, pp. B36-B50.
 DOCUMENT TYPE: Article; Journal
 FILE SEGMENT: LIFE; CLIN
 LANGUAGE: ENGLISH
 REFERENCE COUNT: 46

ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB We investigated incidence, severity, and distribution of coronary atherosclerosis, acute **thrombosis**, and plaque fissuring in ischemic **heart** disease (both unstable-acute syndromes and chronic ischemia) and in nonischemic controls. We also studied the structural, immunohistochemical, and biochemical profile of plaques, with and without **thrombus**, including morphometry, immunophenotyping of inflammatory infiltrates, cytokine presence, and ultrastructural features.

Critical coronary stenosis was almost the rule in both acute and chronic ischemic series (> 90%) whereas it reached 50% in control subjects. **Thrombosis** was principally characteristic of unstable-acute ischemic syndromes (unstable angina, 32%; acute myocardial infarction, 52%; cardiac sudden death, 26%) but was also found in chronic ischemia (stable angina, 12%; ischemic cardiomyopathy, 14%) and in control subjects (4%). Plaque fissuring without **thrombus** occurred in low percentages in lipid-rich, severe eccentric plaques in most series.

Major differences were found between pultaceous-rich versus fibrous plaques rather than between plaques with or without **thrombus**. Pultaceous-rich plaques were frequent in sites of critical stenosis, **thrombosis**, and ulceration. Inflammatory infiltrates, i.e., T cells, macrophages, and a few beta cells, mostly occurred in lipid-rich, plaques unrelated to **thrombus**. In adventitia, infiltrates were a common finding unrelated to any syndrome. Necrotizing cytokines such as alpha-TNF were immunohistochemically detected in macrophages, smooth muscle, and intimal cells and detected by immunoblotting in 67% of pultaceous-rich plaques, either with or without **thrombus**. Immune response mediators such as IL-2 were also expressed in analogous plaques but in a minor percentage (50%-40%). Media were extensively damaged in severely diseased vessels with and without **thrombus**. Ultrastructural study showed that the fibrous cap was either highly cellular or densely fibrillar. Intimal injury with collagen exposure was often associated with platelet adhesion, whereas foamy cell exposure was not.

In conclusion, investigated parameters were essentially similar in plaques, both with and without **thrombus**, whereas major differences were found between pultaceous-rich and fibrous plaques. Since platelets adhere to exposed collagen and not to foam cells, the type of exposed substrates could play a major role in **thrombosis**.

ACCESSION NUMBER: 1995:31070 BIOSIS

DOCUMENT NUMBER: PREV199598045370

TITLE: Plasma Levels of Tumor Necrosis Factor and Endothelial Response in **Patients** with Chronic Arterial Obstructive Disease or Raynaud's Phenomenon.

AUTHOR(S): Cimminiello, Claudio (1); Arpaia, Guido; Toschi, Vincenzo; Rossi, Florica; Aloiso, Manuela; Motta, Adele; Bonfardeci, Giuseppe

CORPORATE SOURCE: (1) Via Stefini 12, 20100 Milan Italy

SOURCE: Angiology, (1994) Vol. 45, No. 12, pp. 1015-1022.

ISSN: 0003-3197.

DOCUMENT TYPE: Article

LANGUAGE: English

AB Tumor necrosis factor alpha (**TNF**-alpha) is a cytokine that affects endothelial cells' function by changing their antithrombotic potential to a net **procoagulant** effect. Only a few data have so far been reported for the pathophysiologic role of **TNF** in vascular diseases in the involvement of microvessels and/or macrovessels and a prothrombotic state. In the present study the authors evaluated plasma **TNF** (and interleukin-1) levels in 20 **patients** with chronic arterial obstructive disease (CAOD) with intermittent claudication and 10 **patients** with more severe disease (pain at rest/skin ulcers). In addition, they studied 10 **patients** with Raynaud's phenomenon (RP), suspected to be secondary to a collagen disease. The control group consisted of 20 subjects matched for sex and age with the three groups of **patients**. **TNF** levels were assayed by enzyme-linked immunosorbent assay. The antigen levels of von Willebrand factor (vWF), tissue plasminogen activator (t-PA), and its inhibitor (PAI) were also determined as markers of release from the endothelium, while the fragment 1+2 of prothrombin (F1+2) and **thrombin**-antithrombin III (TAT) complexes were assessed as indexes of systemic **thrombin** generation. **TNF** levels were significantly higher in both groups of CAOD **patients** than in controls or RP **patients**, and the same was true for vWF t-PA was significantly higher only in the CAOD subjects with more severe disease. No differences among groups were seen in PAI antigen/activity or **thrombin** generation. When data were corrected for age, **TNF** no longer differentiated CAOD **patients** from controls and RP subjects. There were strong direct correlations between **TNF** and age ($r=0.57$, $P=0.0001$); **TNF** and t-PA ($r=0.43$, $P=0.002$); and **TNF** and vWF ($r=0.52$, $P=0.0001$). The association of **TNF** with vWF was independent of other variables. The present study suggests that **TNF** plays an important role in the pathophysiology of arterial diseases of atherosclerotic origin, especially during aging.

ACCESSION NUMBER: 94194909 MEDLINE

DOCUMENT NUMBER: 94194909 PubMed ID: 7511719

TITLE: Cytokines in acute myocardial infarction:
selective increase in circulating tumor necrosis factor,
its soluble receptor, and interleukin-1 receptor
antagonist.

AUTHOR: Latini R; Bianchi M; Correale E; Dinarello C A; Fantuzzi G;
Fresco C; Maggioni A P; Mengozzi M; Romano S; Shapiro L; +

CORPORATE SOURCE: Mario Negri Institute for Pharmacological Research, Milan,
Italy.

SOURCE: JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, (1994 Jan)
23 (1) 1-6.

Journal code: 7902492. ISSN: 0160-2446.

PUB. COUNTRY: United States

DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199405

ENTRY DATE: Entered STN: 19940511

Last Updated on STN: 19980206

Entered Medline: 19940502

AB Cytokines play a pathogenetic role in a variety of infective and inflammatory diseases. In the present study, we had two objectives: (a) to define the kinetics of tumor necrosis factor (**TNF**) in plasma after acute myocardial infarction (AMI) in **patients** treated with early **thrombolysis**, and (b) to measure other cytokines, interleukin-1 (IL-1) and **TNF** receptor antagonists, in plasma. **TNF**-alpha, but not IL-1 beta or IL-8, was present in plasma of 6 of 7 **patients** with severe AMI (Killip class 3 or 4). No **TNF** (< 50 pg/ml) was detected in a group of 11 **patients** with uncomplicated myocardial infarction (Killip class 1) or in control **patients** without AMI. Soluble **TNF** receptor type I and IL-1 receptor antagonist (IL-1Ra) were also significantly increased in the group with severe AMI compared with those with uncomplicated AMI. Circulating **TNF** is increased only in AMI complicated by **heart** failure at hospital admission. This finding may have diagnostic and therapeutic relevance.

L25 ANSWER 14 OF 58 MEDLINE on STN DUPLICATE 4

ACCESSION NUMBER: 96418988 MEDLINE
DOCUMENT NUMBER: 96418988 PubMed ID: 8821773
TITLE: **Thrombolytic** therapy with urokinase reduces increased circulating endothelial adhesion molecules in acute myocardial **infarction**.
AUTHOR: Squadrito F; Saitta A; Altavilla D; Ioculano M; Canale P; Campo G M; Squadrito G; Di Tano G; Mazzu A; Caputi A P
CORPORATE SOURCE: Institute of Pharmacology, University of Messina, Italy.
SOURCE: INFLAMMATION RESEARCH, (1996 Jan) 45 (1) 14-9.
Journal code: 9508160. ISSN: 1023-3830.
PUB. COUNTRY: Switzerland
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199610
ENTRY DATE: Entered STN: 19961219
Last Updated on STN: 20000303
Entered Medline: 19961029

AB The aim was to investigate circulating E-selectin and Intercellular Adhesion Molecule-1 (ICAM-1) in acute myocardial **infarction**. Our study was carried out in 80 **patients**, 40 hospitalized for acute myocardial **infarction** (AMI), 20 suffering from chronic stable angina and 20 healthy control subjects. Samples of venous blood were taken from all **patients** at the moment of hospitalization and after 2, 4, 6, 8, 10, 12 and 24 hours from the **thrombolytic** treatment (AMI + urokinase) or conventional therapy (AMI + nitroglycerin), for the dosage of creatinine kinase (CK) and adhesion molecules. The CK was determined by means of a Hitachi 901 automatic analyser using an enzymatic method (reagents Boheringer-Biochemia, Germany). Soluble E-selectin (sE-selectin) and soluble ICAM-1 (sICAM-1) were measured in the serum using a specific immunoassay (British Biotechnology Products). The serum levels of Tumor Necrosis Factor (**TNF**-alpha) were evaluated using an immunoenzymatic assay to quantitate the serum levels of the cytokine (British Biotechnology Products). **Patients** with acute myocardial **infarction** (AMI) had increased serum levels of soluble E-selectin (sE-selectin; AMI + urokinase = 312 +/- 20 ng/ml; AMI + nitroglycerin = 334 +/- 15 ng/ml) and soluble ICAM-1 (sICAM-1; AMI + urokinase = 629 +/- 30 ng/ml; AMI + nitroglycerin = 655 +/- 25 ng/ml) compared to both **patients** with chronic angina (sE-selectin = 67 +/- 10 ng/ml; sICAM-1 = 230 +/- 20 ng/ml) and healthy control subjects (sE-selectin = 53 +/- 15 ng/ml; sICAM-1 200 +/- 16 ng/ml). Furthermore **patients** with acute myocardial **infarction** also had increased serum levels of Tumor Necrosis Factor (**TNF**-alpha = 309 +/- 10 pg/ml; control subjects = 13 +/- 5 pg/ml). **Thrombolytic** therapy with urokinase (1,000,000 IU as an intravenous bolus for 5 minutes, followed by an infusion of an additional 1,000,000 IU for the following two hours) succeeded in producing reperfusion and reduced the serum levels of sE-selectin (52 +/- 13 ng/ml) and sICAM-1 (202 +/- 31 ng/ml). In contrast **patients** not eligible for **thrombolytic** therapy and therefore treated with conventional therapy (a continuous i.v. infusion of nitroglycerin at the dose of 50 mg/die) did not show any significant reduction in both sE-selectin and sICAM-1 throughout the study. Our results confirm previous experimental data and indicate that adhesion mechanisms supporting leukocyte-endothelium interaction may also be operative in human acute myocardial **infarction**.

L41 ANSWER 13 OF 37 MEDLINE on STN DUPLICATE 3

ACCESSION NUMBER: 95358112 MEDLINE

DOCUMENT NUMBER: 95358112 PubMed ID: 7631597

TITLE: Endogenous cytokine antagonists during myocardial ischemia and **thrombolytic** therapy.

AUTHOR: Airaghi L; Lettino M; Manfredi M G; Lipton J M; Catania A

CORPORATE SOURCE: Third Division of Internal Medicine, Ospedale Maggiore di Milano, Italy.

SOURCE: AMERICAN HEART JOURNAL, (1995 Aug) 130 (2) 204-11.
Journal code: 0370465. ISSN: 0002-8703.

PUB. COUNTRY: United States

DOCUMENT TYPE: (CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals

ENTRY MONTH: 199509

ENTRY DATE: Entered STN: 19950921
Last Updated on STN: 19950921
Entered Medline: 19950905

AB We tested the idea that cytokine antagonists are released during acute myocardial ischemia to counteract proinflammatory effects of cytokines. We investigated changes in plasma concentrations of the anticytokine molecules alpha-melanocyte-stimulating hormone (alpha-MSH), interleukin-1 receptor antagonist (IL-1ra), and soluble **tumor necrosis factor** receptor (sTNFr) in patients with acute myocardial infarction (AMI) or unstable angina (UA). Blood samples were collected at presentation in the coronary care unit, at 3-hour intervals for 24 hours, and daily for 4 days thereafter. There were no significant differences in the concentrations of cytokine antagonists in patients with AMI or UA. However, whereas concentrations of alpha-MSH were increased in early samples of patients with AMI or UA who were treated with a **thrombolytic** agent, they were consistently low in untreated patients. IL-1ra concentrations likewise were greater 3 and 6 hours after treatment in patients who underwent **thrombolysis**, whereas there was no significant difference in plasma sTNFr between the two groups. We suggest that during myocardial ischemia and **thrombolysis** anticytokine molecules released from the injured myocardium become available to reduce inflammation caused by cytokines and other mediators of inflammation.

L41 ANSWER 15 OF 37 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC. on STN
ACCESSION NUMBER: 1996:8096 BIOSIS
DOCUMENT NUMBER: PREV199698580231
TITLE: Soluble tumour necrosis factor
receptors 1 and 2 in patients with acute myocardial
infarction.
AUTHOR(S): Schumacher, M.; Eber, B.; Halwachs, G.; Fruhwald, F. M.;
Zweiker, R.; Zettinig, G.; Pokan, R.; Wilders-Truschnig,
M.; Klein, W.
CORPORATE SOURCE: Dep. Intern. Med., Karl-Franzens-Univ., Graz Austria
SOURCE: European Heart Journal, (1995) Vol. 16, No. ABSTR. SUPPL.,
pp. 42.
Meeting Info.: XVIIth Congress of the European Society of
Cardiology Amsterdam, Netherlands August 20-24, 1995
ISSN: 0195-668X.
DOCUMENT TYPE: Conference
LANGUAGE: English

L41 ANSWER 21 OF 37 SCISEARCH COPYRIGHT 2003 THOMSON ISI on STN
 ACCESSION NUMBER: 93:257823 SCISEARCH
 THE GENUINE ARTICLE: KY052
 TITLE: EOSINOPHIL CATIONIC GRANULE PROTEINS IMPAIR
THROMBOMODULIN FUNCTION - A POTENTIAL MECHANISM
 FOR **THROMBOEMBOLISM** IN HYPEREOSINOPHILIC
HEART-DISEASE
 AUTHOR: SLUNGAARD A (Reprint); VERCELLOTTI G M; TRAN T; GLEICH G
 J; KEY N S
 CORPORATE SOURCE: UNIV MINNESOTA, SCH MED, DEPT MED, BOX 480 UHMC,
 MINNEAPOLIS, MN, 55455 (Reprint); MAYO CLIN & RES FDN,
 DEPT IMMUNOL, ROCHESTER, MN, 55905
 COUNTRY OF AUTHOR: USA
 SOURCE: JOURNAL OF CLINICAL INVESTIGATION, (APR 1993)
 Vol. 91, No. 4, pp. 1721-1730.
 ISSN: 0021-9738.
 DOCUMENT TYPE: Article; Journal
 FILE SEGMENT: LIFE
 LANGUAGE: ENGLISH
 REFERENCE COUNT: 50

ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB **Thromboembolism** is a prominent but poorly understood feature of eosinophilic, or Loeffler's, endocarditis. Eosinophil (EO) specific granule proteins, in particular major basic protein (MBP), accumulate on endocardial surfaces in the course of this disease. We hypothesized that these unusually cationic proteins promote **thrombosis** by binding to the anionic endothelial protein **thrombomodulin** (TM) and impairing its **anticoagulant** activities. We find that MBP potently (IC50 of 1-2 μ M) inhibits the capacity of endothelial cell surface TM to generate the natural **anticoagulant** activated protein C (APC). MBP also inhibits APC generation by purified soluble rabbit TM with an IC50 of 100 nM without altering its apparent K(d) for **thrombin** or K(m) for protein C. This inhibition is reversed by polyanions such as chondroitin sulfate E and heparin. A TM polypeptide fragment comprising the extracellular domain that includes its naturally occurring anionic glycosaminoglycan (GAG) moiety (TMD-105) is strongly inhibited by MBP, whereas its counterpart lacking the GAG moiety (TMD-75) is not. MBP also curtails the capacity of TMD-105 but not TMD-75 to prolong the **thrombin clotting** time. Thus, EO cationic proteins potently inhibit **anticoagulant** activities of the glycosylated form of TM, thereby suggesting a potential mechanism for **thromboembolism** in hypereosinophilic heart disease.

L5 ANSWER 53 OF 62 MEDLINE
 ACCESSION NUMBER: 84060862 MEDLINE
 DOCUMENT NUMBER: 84060862 PubMed ID: 6643010
 TITLE: Blood coagulation alterations and **thromboembolism**
 in **Crohn's** disease.
 AUTHOR: Leardi S; Amoroso A; Afeltra A; Ferri G M; Tebano M T;
 Simi
 M; Speranza
 SOURCE: ITALIAN JOURNAL OF SURGICAL SCIENCES, (1983) 13
 (3) 197-201.
 Journal code: GY; 8213451. ISSN: 0392-3525.
 PUB. COUNTRY: Italy
 Journal; Article; (JOURNAL ARTICLE)
 LANGUAGE: English
 FILE SEGMENT: Priority Journals
 ENTRY MONTH: 198401
 ENTRY DATE: Entered STN: 19900319
 Last Updated on STN: 19900319
 Entered Medline: 19840107

AB The present study was undertaken in view of the higher incidence of
thromboembolism in patients with **Crohn's** disease. The
 blood coagulation system was studied in 12 patients previously operated
 for Crohn's disease (8 cases of ileitis, 4 cases of colitis) and followed
 as out-patients. In 75% of cases, the disease was in an inactive stage.
 Eight patients showed slight lipid malabsorption. Serum levels of
 fibrinogen, platelets and factor V were shown to be significantly
 increased (p less than 0.001) as compared to controls. Prothrombin time
 and factors II, VII and X were shown to be decreased, while factors VIII
 and IX and antithrombin III were not significantly altered.

Thrombocytosis
 and hyperfibrinogenemia, as reported in literature, seem to determine a
 condition of blood hypercoagulability, playing therefore a primary
 pathogenetic role in the genesis of **thromboembolism** in patients
 with **Crohn's** disease.

L18 ANSWER 7 OF 8 SCISEARCH COPYRIGHT 2003 THOMSON ISI on STN

ACCESSION NUMBER: 96:91926 SCISEARCH

THE GENUINE ARTICLE: TQ489

TITLE: RELEASE OF **THROMBOMODULIN** FROM ENDOTHELIAL-CELLS
BY CONCERTED ACTION OF **TNF-ALPHA** AND NEUTROPHILS
- IN-VIVO AND IN-VITRO STUDIES

AUTHOR: BOEHME M W J (Reprint); DENG Y; RAETH U; BIERHAUS A;
ZIEGLER R; STREMMEL W; NAWROTH P P

CORPORATE SOURCE: UNIV HEIDELBERG, DEPT INTERNAL MED 4, BERGHEIMER STR 58,
D-69115 HEIDELBERG, GERMANY (Reprint); UNIV HEIDELBERG,
DEPT INTERNAL MED 1, D-69115 HEIDELBERG, GERMANY

COUNTRY OF AUTHOR: GERMANY

SOURCE: IMMUNOLOGY, (JAN 1996) Vol. 87, No. 1, pp. 134-140.
ISSN: 0019-2805.

DOCUMENT TYPE: Article; Journal

FILE SEGMENT: LIFE

LANGUAGE: ENGLISH

REFERENCE COUNT: 63

ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Inflammatory cytokines decrease the expression of thrombomodulin (TM) on the endothelial cell surface by suppression of TM transcription and translation or internalization with subsequent degradation. Nevertheless, elevated serum TM levels are found in diseases associated with systemical or locally increased levels of inflammatory cytokines. To study directly the in vivo effects of tumour necrosis factor-alpha (TNF-alpha) we determined the course of serum TM after systemic recombinant human (rh)TNF-alpha therapy. The TM levels were determined by enzyme-linked immunosorbent assay (ELISA). Systemic rhTNF-alpha therapy resulted in a marked and significant increase of serum TM. Using a mouse model we studied whether increased serum TM is associated with a decreased expression of TM on the endothelial surface in vivo. The immunohistochemical staining of the vasculature of meth-A sarcoma transplanted in mice showed a loss of TM immunoreactivity 4 hr after intravenous TNF-alpha application. To study the mechanism of TNF-alpha mediated release of TM, cultured endothelial cells were incubated with neutrophils and TNF-alpha. Incubation with TNF-alpha alone did not lead to an increase of TM in vitro. However TM was released into the culture supernatant when endothelial cells pretreated with TNF-alpha were exposed to neutrophils. This was associated with morphological evidence of endothelial cell damage. Therefore, the concerted action of cytokine-stimulated endothelial cells and neutrophils results in release of TM from cultured endothelial cells after rhTNF-alpha therapy. This might explain the increased serum TM levels observed in diseases associated with increased systemic or local levels of inflammatory cytokines despite the induced internalization and the direct inhibitory effects of TNF-alpha on TM transcription and translation.

L10 ANSWER 13 OF 28

MEDLINE on STN

DUPLICATE 4

ACCESSION NUMBER: 96302769 MEDLINE

DOCUMENT NUMBER: 96302769 PubMed ID: 8743182

TITLE: Infusion of phospholipid vesicles amplifies the local thrombotic response to TNF and anti-protein C into a consumptive response.

AUTHOR: Taylor F B Jr; He S E; Chang A C; Box J; Ferrell G; Lee D; Lockhart M; Peer G; Esmon C T

CORPORATE SOURCE: Oklahoma Medical Research Foundation, Oklahoma City 73104, USA.

CONTRACT NUMBER: 2R01 GM37704 (NIGMS)

R37 HL30340 (NHLBI)

SOURCE: THROMBOSIS AND HAEMOSTASIS, (1996 Apr) 75 (4) 578-84.

Journal code: 7608063. ISSN: 0340-6245.

PUB. COUNTRY: GERMANY: Germany, Federal Republic of

DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199611

ENTRY DATE: Entered STN: 19961219

Last Updated on STN: 19961219

Entered Medline: 19961105

AB Inflammation often is considered a contributing factor to both thrombosis and disseminated intravascular coagulation. The molecular mechanisms that dictate which of these clinical manifestations will result from the inflammatory stimulus remain obscure. Bacterial infection and certain tumors are common initiators of the disseminated intravascular coagulant response. Complement activation resulting from bacterial infection shares with selected tumors the capacity to generate or release membrane particles that lack functional adhesion receptors and hence could circulate to amplify a disseminated intravascular coagulant response. We developed a model of venous thrombosis that resulted in localized thrombus formation without disseminated intravascular coagulation. The model involves infusion of tumor necrosis factor, blockade of protein C and a partial decrease in venous flow caused by ligation of the superficial femoral vein without obstruction of the deep femoral vein. Infusion of phospholipid vesicles into this model resulted in amplification of a localized thrombotic response into a consumptive response. Seven different groups of animals were studied. The first three groups established the conditions necessary to produce deep vein thrombosis. The second four groups established the conditions necessary to produce disseminated intravascular coagulation. The infusion of phospholipid vesicles plus **tumor necrosis factor** and anti-protein C **antibody** resulted in consumption of **fibrinogen**, the production of thrombin/antithrombin complexes, a fall in platelet count, and venous thrombosis. Without ligation and catheterization phospholipid vesicles failed to produce the consumptive response. We conclude, therefore, that phospholipid vesicles can amplify a local thrombotic response into a consumptive response, and that vesiculation accompanying inflammation is one means by which localized coagulant activity may be amplified to produce disseminated intravascular coagulation.

L10 ANSWER 18 OF 28 MEDLINE on STN DUPLICATE 7

ACCESSION NUMBER: 93085922 MEDLINE
DOCUMENT NUMBER: 93085922 PubMed ID: 1453598
TITLE: Procoagulant effect of the OKT3 monoclonal antibody
: involvement of tumor necrosis
factor.
AUTHOR: Pradier O; Marchant A; Abramowicz D; De Pauw L;
Vereerstraeten P; Kinnaert P; Vanherweghem J L; Capel P;
Goldman M
CORPORATE SOURCE: Department of Immunology, Hematology and Transfusion,
Hopital Erasme, Universite Libre de Bruxelles, Belgium.
SOURCE: KIDNEY INTERNATIONAL, (1992 Nov) 42 (5) 1124-9.
Journal code: 0323470. ISSN: 0085-2538.
PUB. COUNTRY: United States
DOCUMENT TYPE: (CLINICAL TRIAL)
(CONTROLLED CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199301
ENTRY DATE: Entered STN: 19930129
Last Updated on STN: 19990129
Entered Medline: 19930107

AB We recently observed that the prophylactic administration of high doses of OKT3 monoclonal antibody (MoAb) in cadaveric renal transplantation favors the development of thromboses of the grafts' main vessels and of thrombotic microangiopathies. These clinical observations led us to perform sequential determinations of plasma levels of prothrombin fragment 1 and 2 (F 1 + 2) and fibrin degradation products (FDP) after the first injection of 5 or 10 mg OKT3 given as prophylaxis in kidney transplant recipients. The values observed have been compared with those of kidney transplant recipients not treated with OKT3. F 1 + 2 levels peaked four hours after the first injection of 5 mg OKT3 (mean +/- SEM: 4.82 +/- 0.73 vs. 1.75 +/- 0.37 nmol/liter in controls, $P < 0.01$), indicating activation of the common pathway of the coagulation cascade. FDP levels were already above baseline values at four hours and continued to increase until 24 hours (mean +/- SEM at 24 hr, 4729 +/- 879 vs. 1038 +/- 320 ng/ml in controls, $P < 0.05$), indicating a fibrinolytic process. The magnitude and the time course of the changes in F 1 + 2 and FDP plasma levels were similar whether the patients received 5 or 10 mg dose of OKT3. The levels of von Willebrand factor (VWF) antigen, a molecule released by activated or damaged endothelial cells, were also significantly increased after injection of OKT3 (mean +/- SEM at 24 hr, 3.67 +/- 0.18 vs. 2.17 +/- 0.11 U/ml in controls, $P < 0.05$). The procoagulant effects of OKT3 were further investigated in vitro on human umbilical vein endothelial cells (HUVEC). (ABSTRACT TRUNCATED AT 250 WORDS)

L10 ANSWER 20 OF 28 MEDLINE on STN DUPLICATE 9
ACCESSION NUMBER: 91126052 MEDLINE
DOCUMENT NUMBER: 91126052 PubMed ID: 1671533
TITLE: CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the A alpha chain of **fibrinogen**.
AUTHOR: Loiike J D; Sodeik B; Cao L; Leucona S; Weitz J I; Detmers P A; Wright S D; Silverstein S C
CORPORATE SOURCE: Columbia University, New York, NY 10032.
CONTRACT NUMBER: AI22003 (NIAID)
DK39110 (NIDDK)
GM40791 (NIGMS)

+
SOURCE: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1991 Feb 1) 88 (3) 1044-8.
Journal code: 7505876. ISSN: 0027-8424.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199103
ENTRY DATE: Entered STN: 19910405
Last Updated on STN: 19950206
Entered Medline: 19910308

AB **Fibrinogen** and fibrin serve as adhesive substrates for a variety of cells including platelets, endothelial cells, and leukocytes. Previously, we identified the C terminus of the gamma chain of **fibrinogen** as the region of the **fibrinogen** molecule that contains a ligand for CD11b/CD18 (complement receptor 3) on phorbol ester-stimulated polymorphonuclear leukocytes. In contrast, we report here that neutrophils stimulated with tumor necrosis factor adhere to **fibrinogen**-coated surfaces, but not to human serum albumin-coated surfaces, via the integrin CD11c/CD18 (p150/95). Monoclonal antibodies LeuM5 and 3.9, which are directed against the alpha subunit of CD11c/CD18, but not monoclonal antibodies OKM10 and OKM1, which are directed against the alpha subunit of CD11b/CD18, inhibit the adhesion of tumor necrosis factor-stimulated neutrophils to **fibrinogen**-coated surfaces. To identify the site on **fibrinogen** recognized by CD11c/CD18, we have examined the adhesion of tumor necrosis factor-stimulated neutrophils to surfaces coated with various **fibrinogen** fragments. Stimulated neutrophils adhere to surfaces coated with the N-terminal disulfide knot fragment of **fibrinogen** or **fibrinogen** fragment E. Moreover, peptides containing the sequence Gly-Pro-Arg (which corresponds to amino acids 17-19 of the N-terminal region of the A alpha chain of **fibrinogen**), and monoclonal antibody LeuM5, block tumor necrosis factor-stimulated neutrophil adhesion to **fibrinogen** and to the N-terminal disulfide knot fragment of **fibrinogen**. Thus, CD11c/CD18 on tumor necrosis factor-stimulated neutrophils functions as a **fibrinogen** receptor that recognizes the sequence Gly-Pro-Arg in the N-terminal domain of the A alpha chain of **fibrinogen**.

L10 ANSWER 22 OF 28 MEDLINE on STN DUPLICATE 10

ACCESSION NUMBER: 90354031 MEDLINE
DOCUMENT NUMBER: 90354031 PubMed ID: 2201637
TITLE: Tumor necrosis factor (TNF) is induced in mice by Candida albicans: role of TNF in **fibrinogen** increase.
AUTHOR: Riipi L; Carlson E
CORPORATE SOURCE: Department of Biological Sciences, Michigan Technological University, Houghton 49931.
SOURCE: INFECTION AND IMMUNITY, (1990 Sep) 58 (9) 2750-4.
Journal code: 0246127. ISSN: 0019-9567.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199009
ENTRY DATE: Entered STN: 19901026
Last Updated on STN: 19901026
Entered Medline: 19900927

AB One intraperitoneal dose of Candida albicans (10(8) CFU) caused a chronic (longer than 2 months), significant elevation of plasma **fibrinogen** levels (Clauss method) in mice of strain C3H/HeN. Even a small dose (10(6) CFU) resulted in a significant increase in **fibrinogen** level for 5 days following injection, whereas other blood parameters (leukocytes, erythrocytes, platelets, hematocrit, hemoglobin, blood urea nitrogen, aspartate aminotransferase, albumin, alkaline phosphatase, antithrombin III, glucose, calcium, and total protein) measured by standard methods were normal. Blood taken during this period was negative for C. albicans. The role of tumor necrosis factor (TNF) in C. albicans infections was investigated by measuring the **fibrinogen** response after the administration of C. albicans or recombinant mouse TNF-alpha. Both challenges resulted in an elevated **fibrinogen** level. When polyclonal **antibodies** to mouse **TNF**-alpha were given prior to challenge with C. albicans or mouse TNF-alpha, the **fibrinogen** increase was significantly inhibited. C. albicans injections were found to significantly elevate endogenous TNF levels in mice (enzyme-linked immunosorbent assay). It was concluded that C. albicans induces TNF in the mouse. Furthermore, these data give evidence which supports a relationship between TNF and the **fibrinogen** increase induced by C. albicans.

L16 ANSWER 18 OF 21 PCTFULL COPYRIGHT 2003 Univentio on STN
ACCESSION NUMBER: 1992003145 PCTFULL ED 20020513
TITLE (ENGLISH): METHOD OF TREATING VIRAL INFECTION
TITLE (FRENCH): PROCEDE DE TRAITEMENT DES INFECTIONS VIRALES
INVENTOR(S): RATHJEN, Deborah, Ann;
ASTON, Roger;

PATENT ASSIGNEE(S): RAMSHAW, Ian, Alastair
PEPTIDE TECHNOLOGY LTD.;
RATHJEN, Deborah, Ann;
ASTON, Roger;
RAMSHAW, Ian, Alastair

LANGUAGE OF PUBL.: English

DOCUMENT TYPE: Patent

PATENT INFORMATION:

NUMBER	KIND	DATE
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WO 9203145	A1	19920305
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DESIGNATED STATES

W:

AT AU BE CA CH DE DK ES FR GB GR IT JP LU NL SE US

APPLICATION INFO.:

WO 1991-AU400 A 19910827

PRIORITY INFO.:

AU 1990-PK 1976 19900827

DETD 5 Incorporation of 125I **Fibrinogen** into Tumours of Mice
Treated with **TNF** and Monoclonal **Antibody**
In order to examine the effect of **TNF** and monoclonal
antibodies on fibrin formation in vivo, BALB/c mice were
injected subcutaneously with WEHI-164 cells (105
cells/animal). After 7 - 14 days, when tumours reached a
size of approximately 1 cm in diameter, animals were
injected intra-peritoneally with **TNF** (10 ug/animal) and
125I human **fibrinogen** (7.5ug/animal, 122uCi/mg Amersham)
either alone or in the presence of monoclonal **antibody** to
human **TNF** (200ul/animal ascitic globulin). Monoclonal
antibody against bovine growth hormone was used as control
monoclonal **antibody**. Two hours after **TNF** infusion
incorporation of 125I **fibrinogen** into mouse tissue was
determined by removing a piece of tissue, weighing it and
counting the sample in a gamma counter.

L16 ANSWER 19 OF 21 PCTFULL COPYRIGHT 2003 Univentio on STN
 ACCESSION NUMBER: 1991002078 PCTFULL ED 20020513
 TITLE (ENGLISH): TUMOUR NECROSIS FACTOR BINDING LIGANDS
 TITLE (FRENCH): LIGANDS DE LIAISON DU FACTEUR-DE NECROSE DE TUMEURS
 INVENTOR(S): RATHJEN, Deborah, Anne;
 ASTON, Roger
 PATENT ASSIGNEE(S): PEPTIDE TECHNOLOGY LTD;
 RATHJEN, Deborah, Anne;
 ASTON, Roger
 LANGUAGE OF PUBL.: English
 DOCUMENT TYPE: Patent
 PATENT INFORMATION:

NUMBER	KIND	DATE
WO 9102078	A1	19910221

DESIGNATED STATES

W:	AT AU BE CA CH DE DK ES FR GB IT JP LU NL SE US
APPLICATION INFO.:	WO 1990-AU337 A 19900807
PRIORITY INFO.:	AU 1989-PJ 5662 19890807
	AU 1989-PJ 7576 19891124

DETD . . . a titration assay with
 MAb I against TNF,,
 Fig. 2 shows TNF MAb 1 scatchard plot and affinity
 deternlnatign;
 F.ig. 3 shows the@effect of anti-TNF monoclonal
antibodies I and 32 on **TNF** cytotoxicity in WEHI-164
 cells;
 Fig.-4 shows the effect of MAb 1 on TNF-induced
 regression of a Meth A solid-tumour;
 Fig-6 5 shows.the effect. . . regression;
 Fig, 6-shows the effect of anti-TNF MAb on induction
 of endothelial cell procoagulant activity by TNF;
 Fig. 7 shows incorporation of labelled **fibrinogen**
 into tumours of tumour-bearing mice and the effect of
 anti-TNF mAbs,*
 Fig. 8 is a schematic representation of epitopes on
 TNF;
 Fig. 9 shows the. . . MAb 32 (- 4), control antibody
 and MAb 47 on melanoma cell line MM418E;
 Fig4 14 shows receptor binding studies of **TNF**
 complexed with MAb 32 control **antibody**
 and MAb 47 (--A*--) on melanoma cell line IGR3;
 Fig. 15 shows receptor binding studies of **TNF**
 complexed with MAb 32 control **antibody**
 -
 and MAb 47 on bladder carcinoma cell line 5637;
 Fig. 16 shows receptor binding studies of **TNF**
 complexed with MAb 32 control **antibody**
 and MAb 47 on breast carcinoma cell line MCF7;
 Fig. 17 shows receptor binding studies of **TNF**
 complexed with MAb 32 (#), control **antibody**
 and MAb 47 (--*&--) on colon carcinoma cell line BIO;
 Fig. 18 shows the effect on TNF-mediated tumour
 regression in vivo by MAb. . .
 .
 (30M) and the time taken
 for clot formation recorded. In some experiments tumour
 cell culture supernatant was added to endothelial cells
 treated with **TNF** and/or monoclonal **antibody** (final
 concentration of 1 in 2),
 Incorporation of 125I **Fibrinogen** into Tumours of Mice
 Treated with TNF and Monoclonal Antibo@y

In order to examine the effect of **TNF** and monoclonal **antibodies** on fibrin formation in vivo, BALB/c mice were

injected subcutaneously with WEHI-164 cells (10⁵ cells/animal). After 7 - 14 days, when tumours reached a size of approximately 1 cm in diameter, animals were injected intra-peritoneally with **TNF** (10 ug/animal) and 5 125I human **fibrinogen** (7.5ug/animal, 122uCi/mg Amersham) either alone or in the presence of monoclonal **antibody** to human **TNF** (200ul/animal ascitic globulin). monoclonal

antibody against bovine growth hormone was used as control monoclonal **antibody**. Two hours after **TNF** infusion incorporation of 125I **fibrinogen** into mouse tissue was determined by removing a piece of tissue, weighing it and counting the sample in a gamma counter.

L24 ANSWER 1 OF 3 USPATFULL on STN

ACCESSION NUMBER: 97:56799 USPATFULL
TITLE: Tumour necrosis factor binding ligands
INVENTOR(S): Rathjen, Deborah Ann, New South Wales, Australia
Aston, Roger, Gloucester, Great Britain
PATENT ASSIGNEE(S): Peptide Technology Ltd., Australia (non-U.S.
corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5644034		19970701
APPLICATION INFO.:	US 1994-344133		19941123 (8) <--
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1992-828956, filed on 18 Feb 1992, now abandoned		

	NUMBER	DATE
PRIORITY INFORMATION:	AU 1989-5662	19890807
	AU 1989-7576	19891124
	WO 1990-AU337	19900807
DOCUMENT TYPE:	Utility	
FILE SEGMENT:	Granted	
PRIMARY EXAMINER:	Eisenschenk, Frank C.	
LEGAL REPRESENTATIVE:	Banner & Witcoff, Ltd.	
NUMBER OF CLAIMS:	7	
EXEMPLARY CLAIM:	1	
NUMBER OF DRAWINGS:	41 Drawing Figure(s); 28 Drawing Page(s)	
LINE COUNT:	1493	

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AI US 1994-344133 19941123 (8) <--

DETD Incorporation of 125I **Fibrinogen** into Tumours of Mice Treated
with **TNF** and Monoclonal **Antibody**

DETD In order to examine the effect of **TNF** and monoclonal
antibodies on fibrin formation in vivo, BALB/c mice were
injected subcutaneously with WEHI-164 cells (10.sup.5 cells/animal).
After 7-14 days, when tumours. . . reached a size of approximately 1
cm in diameter, animals were injected intra-peritoneally with **TNF** (10
ug/animal) and 125I human **fibrinogen** (7.5 ug/animal, 122
uCi/mg Amersham) either alone or in the presence of monoclonal
antibody to human **TNF** (200ul/animal ascitic globulin).
Monoclonal **antibody** against bovine growth hormone was used as
control monoclonal **antibody**. Two hours after **TNF**
infusion incorporation of 125I **fibrinogen** into mouse tissue
was determined by removing a piece of tissue, weighing it and counting
the sample in a gamma. . .

ACCESSION NUMBER: 659427 EUROPATFULL EW 200230 FS PS
 TITLE: 2-Phenyl-3-azoylbenzothiophenes for increasing
 thrombomodulin expression.
 2-Phenyl-3-Azoylbenzothiophene zur Erhoehung der
 Thrombomodulin Expression.
 2-Phenyl-3-azoylbenzothiophenes pour augmenter
 l'expression de la thrombomoduline.
 INVENTOR(S): Calnek, David Scott, 7040 East Doral North Drive,
 Indianapolis, Indiana 46250, US;
 Grinnell, Brian William, 3625 East 71st Street,
 Indianapolis, Indiana 46220, US
 PATENT ASSIGNEE(S): ELI LILLY AND COMPANY, Lilly Corporate Center,
 Indianapolis, Indiana 46285, US
 PATENT ASSIGNEE NO: 204942
 AGENT: Vaughan, Jennifer Ann et al., Eli Lilly and Company
 Limited European Patent Operations Erl Wood Manor,
 Windlesham, Surrey GU20 6PH, GB
 AGENT NUMBER: 84383
 OTHER SOURCE: BEPB2002051 EP 0659427 B1 0011
 SOURCE: Wila-EPS-2002-H30-T1
 DOCUMENT TYPE: Patent
 LANGUAGE: Anmeldung in Englisch; Veroeffentlichung in Englisch
 DESIGNATED STATES: R AT; R BE; R CH; R DE; R DK; R ES; R FR; R GB; R GR; R
 IE; R IT; R LI; R LU; R NL; R PT; R SE; R LT; R SI
 PATENT INFO.PUB.TYPE: EPB1 EUROPAEISCHE PATENTSCHRIFT
 PATENT INFORMATION:

PATENT NO	KIND	DATE
EP 659427	B1	20020724
		19950628
EP 1994-309485		19941219
US 1993-170944		19931221
US 4418068	A	

'OFFENLEGUNGS' DATE: JOURNAL OF MEDICINAL CHEMISTRY, vol. 27, no.8, 1984
 APPLICATION INFO.: pages 1057-1066, C.D. JONES ET AL. 'ANTIESTROGENS.2.'
 PRIORITY APPLN. INFO.: JAMA, vol. 243, no.6, 1980 pages 514-515, A. HENDRICK ET
 REFERENCE PAT. INFO.: AL. 'TAMOXIFEN AND THROMBOEMBOLISM'
 REF. NON-PATENT-LIT.:

✓

L5 ANSWER 53 OF 62 MEDLINE
ACCESSION NUMBER: 84060862 MEDLINE
DOCUMENT NUMBER: 84060862 PubMed ID: 6643010
TITLE: Blood coagulation alterations and **thromboembolism**
in **Crohn's** disease.
AUTHOR: Leardi S; Amoroso A; Afeltra A; Ferri G M; Tebano M T;
Simi
M; Speranza
SOURCE: ITALIAN JOURNAL OF SURGICAL SCIENCES, (1983) 13
(3) 197-201.
Journal code: GYY; 8213451. ISSN: 0392-3525.
PUB. COUNTRY: Italy
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 198401
ENTRY DATE: Entered STN: 19900319
Last Updated on STN: 19900319
Entered Medline: 19840107

AB The present study was undertaken in view of the higher incidence of **thromboembolism** in patients with **Crohn's** disease. The blood coagulation system was studied in 12 patients previously operated for Crohn's disease (8 cases of ileitis, 4 cases of colitis) and followed as out-patients. In 75% of cases, the disease was in an inactive stage. Eight patients showed slight lipid malabsorption. Serum levels of fibrinogen, platelets and factor V were shown to be significantly increased (p less than 0.001) as compared to controls. Prothrombin time and factors II, VII and X were shown to be decreased, while factors VIII and IX and antithrombin III were not significantly altered.

Thrombocytosis
and hyperfibrinogenemia, as reported in literature, seem to determine a condition of blood hypercoagulability, playing therefore a primary pathogenetic role in the genesis of **thromboembolism** in patients with **Crohn's** disease.

L18 ANSWER 8 OF 8

MEDLINE on STN

DUPLICATE 4

ACCESSION NUMBER: 96400093 MEDLINE
DOCUMENT NUMBER: 96400093 PubMed ID: 8806469
TITLE: P-selectin and **TNF** inhibition reduce venous
thrombosis inflammation.
AUTHOR: Wakefield T W; Strieter R M; Downing L J; Kadell A M; Wilke
C A; Burdick M D; Wroblewski S K; Phillips M L; Paulson J C;
Anderson D C; Greenfield L J
CORPORATE SOURCE: Department of Surgery, University of Michigan Medical
Center, Ann Arbor 48109, USA.
CONTRACT NUMBER: HL50057 (NHLBI)
HL53355 (NHLBI)
SOURCE: JOURNAL OF SURGICAL RESEARCH, (1996 Jul 15) 64 (1) 26-31.
Journal code: 0376340. ISSN: 0022-4804.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199612
ENTRY DATE: Entered STN: 19970128
Last Updated on STN: 19970128
Entered Medline: 19961205

AB Venous thrombosis induces a detrimental inflammatory response in the vein wall. The cytokine tumor necrosis factor-alpha (TNF) and the adhesion molecules, selectins, have been found to be important in mediating inflammatory cell stimulation and leukocyte-endothelial cell adhesion, respectively. This study assesses the role of TNF and P-selectin in the inflammatory events associated with venous thrombosis. Rats were passively immunized with neutralizing anti-TNF serum alone, anti-TNF plus anti-P-selectin **antibody**, anti-P-selectin **antibody** alone, control serum, or control anti-P-selectin **antibody**. **Antibodies** or control sera were given prior to occlusion and at Days 2 and 4 postocclusion. Rats were sacrificed at Days 1-6 and Day 13 after occlusion for inferior vena caval (IVC) wall histopathology and TNF analysis. Differences in the extent of inflammatory cell infiltrate into the vein wall were found on Days 2, 6, and 13. TNF levels were elevated in the vein wall of the three groups not given anti-TNF **antibody**. The levels of TNF at Day 6 positively correlated with both total inflammatory cell ($r = 0.53$, $P < 0.05$) and neutrophil presence ($r = 0.72$, $P < 0.01$). The lowest IVC wall neutrophil and total inflammatory cell count at Days 2 and 6 and the lowest neutrophil count at Day 13 were found in the anti-TNF plus anti-P-selectin **antibody** group. Monocyte influx was also inhibited at Day 13 in this group. These results suggest a role for combined neutralization of TNF and P-selectin in the attenuation of inflammation induced by venous thrombosis.

L18 ANSWER 4 OF 8 BIOTECHDS COPYRIGHT 2003 THOMSON DERWENT/ISI on STN

ACCESSION NUMBER: 2002-10908 BIOTECHDS

TITLE: New **antibody** specific for human tumor necrosis factor (TNF)-alpha; useful for treating TNF -alpha-mediated diseases, e.g. congestive heart failure, septic or endotoxic shock, cachexia, adult respiratory distress syndrome;
vector plasmid pTTO-mediated gene transfer and expression in Escherichia coli and Fab **antibody** for use in rheumatoid arthritis, osteoarthritis, cardiovascular disease, respiratory disease, AIDS, allergy, psoriasis, tuberculosis, inflammatory bone disorder, blood coagulation disorder, autoimmune disease, vulnerary and transplantation therapy

AUTHOR: ATHWAL D S; BROWN D T; WEIR A N C; POPPLEWELL A G; CHAPMAN A P; KING D J

PATENT ASSIGNEE: CELLTECH R and D LTD

PATENT INFO: WO 2001094585 13 Dec 2001

APPLICATION INFO: WO 2000-GB2477 6 Jun 2000

PRIORITY INFO: GB 2000-13810 6 Jun 2000

DOCUMENT TYPE: Patent

LANGUAGE: English

OTHER SOURCE: WPI: 2002-216732 [27]

AN 2002-10908 BIOTECHDS

AB DERWENT ABSTRACT:

NOVELTY - An **antibody** molecule having specificity for human tumor necrosis factor-alpha (TNFalpha), comprising a heavy or light chain, is new.

DETAILED DESCRIPTION - An **antibody** molecule having

L30 ANSWER 5 OF 6 MEDLINE on STN DUPLICATE 4

ACCESSION NUMBER: 94201038 MEDLINE
DOCUMENT NUMBER: 94201038 PubMed ID: 8150660
TITLE: Ultrastructural localization of tumour necrosis factor-alpha.
AUTHOR: Schmauder-Chock E A; Chock S P; Patchen M L
CORPORATE SOURCE: Department of Experimental Hematology, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20889-5603.
SOURCE: HISTOCHEMICAL JOURNAL, (1994 Feb) 26 (2) 142-51.
Journal code: 0163161. ISSN: 0018-2214.
PUB. COUNTRY: ENGLAND: United Kingdom
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199405
ENTRY DATE: Entered STN: 19940523
Last Updated on STN: 19940523
Entered Medline: 19940512

AB The application of an antibody against tumour necrosis factor-alpha (**TNF**) to thin sections of plastic-embedded mouse tissue has identified sites of **TNF** activity in normal and endotoxin-treated C3N/HeN mice. Prior to endotoxin treatment, **TNF** was observed in the secretory granules of the antibacterial Paneth cell and one type of crypt endocrine cell. Four hours after endotoxin treatment, these two types of intestinal cell were found to have degranulated. In addition, endotoxin treatment resulted in the appearance of **TNF** in the secretory granules of all eosinophils, neutrophils and monocytes in the bone marrow, spleen, lung and the proximal intestine. **TNF** was also observed in the internal elastic lamina (IEL) of arterioles. These results suggest that the process of **TNF** induction specifically targets the immune system and the vasculature. An invasive stimulus, such as circulating endotoxin, can provoke the immune cells to be armed with **TNF**. That same stimulus may cause arteriole smooth muscle cells to secrete **TNF**. **TNF** secretion in the presence of arteriole smooth muscle cells may play a role in the adjustment of arteriole tone. In the venules, **TNF** may be responsible for platelet and neutrophil accumulation which leads to **embolism** formation.

L25 ANSWER 9 OF 58

MEDLINE on STN

DUPLICATE 3

ACCESSION NUMBER: 97415829 MEDLINE

DOCUMENT NUMBER: 97415829 PubMed ID: 9270121

TITLE: The effect of reperfusion on plasma tumor necrosis factor alpha and C reactive protein levels in the course of acute myocardial infarction.

AUTHOR: Pudil R; Pidrman V; Krejsek J; Gregor J; Tichy M; Andrys C; Drahosova M

CORPORATE SOURCE: IInd Dept. of Medicine, Charles University, Faculty of Medicine, Hradec Kralove.

SOURCE: ACTA MEDICA (HRADEC KRALOVE), (1996) 39 (4) 149-53.

Journal code: 9705947. ISSN: 1211-4286.

PUB. COUNTRY: Czech Republic

DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199709

ENTRY DATE: Entered STN: 19971008

Last Updated on STN: 19971008

Entered Medline: 19970925

AB Severe acute response, the synthesis of human acute-phase proteins and the increase of plasma cytokines and adhesion molecules occur in **patients** in the course of acute myocardial infarction.

We examined the plasma tumor necrosis factor alpha (**TNF** alpha), plasma creatinkinase (CK) and C-reactive protein (C-RP) levels in **patients** with acute myocardial infarction (AMI) in the course of 96 hours. Venous blood samples were taken at 3-hour intervals during the first 48 hours, and at 6-hour intervals during the next 48 hours. All **patients** were treated using thrombolytic therapy (streptokinase). Detection of the reperfusion was based on the method of measuring the time to achieve peak serum creatinkinase activity. The study was done on a group of 24 **patients**. Plasma levels of the parameters were compared between the group of **patients** with expected reperfusion versus the group of **patients** in which reperfusion is not suggested. The plasma **TNF** alpha level was elevated constantly without any significant peak. The mean plasma **TNF** alpha concentration was 46.8 pg/ml, SD 2.13, vs. normal level 4.35 pg/ml, $p < 0.001$. The plasma **TNF** alpha level in the group of **patients** with reperfused coronary artery showed a significant decrease especially during the 3rd and 4th day (the mean peak plasma **TNF** alpha concentration was 35.2 pg/ml, SD 15.8, vs. 66.9 pg/ml, SD 38.3 pg/ml, $p < 0.005$). The plasma C-RP levels were elevated throughout the time of observation in the both groups. The elevation of the plasma C-RP levels was more significant in the group of **patients** without successful reperfusion (80.6 mg/ml, SD 31.2, the mean plasma C-RP level of the group of the **patients** with successful reperfusion was 45.7 mg/ml, SD 18.1, $p < 0.005$). We conclude, that **TNF** alpha can play a role in the mechanisms of tissue injury. The successful reperfusion of coronary artery leads to significant decrease of plasma **TNF** alpha and C-RP levels.

L25 ANSWER 50 OF 58 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC. on STN
ACCESSION NUMBER: 1993:11575 BIOSIS
DOCUMENT NUMBER: PREV199395011575
TITLE: Interleukin-2 induces activation of **coagulation**
and fibrinolysis: Resemblance to the changes seen during
experimental endotoxaemia.
AUTHOR(S): Baars, Johanna W.; De Boer, Jan Paul; Wagstaff, John (1);
Roem, Dorina; Eerenberg-Belmer, Anke J. M.; Nauta, Jos;
Pinedo, Herbert M.; Hack, C. Erik
CORPORATE SOURCE: (1) Dep. Medical Oncology, Free University Hospital, De
Boelelaan 1117, 1081 HV Amsterdam Netherlands Antilles
SOURCE: British Journal of Haematology, (1992) Vol. 82, No. 2, pp.
295-301.
ISSN: 0007-1048.
DOCUMENT TYPE: Article
LANGUAGE: English

AB The administration of Interleukin-2 (IL-2) causes the release or
generation of other cytokines such as tumour necrosis factor (TNF
) which, by disturbing the **anticoagulant** properties of the
endothelium, may induce a **procoagulant** state in **patients**
receiving this drug. We therefore evaluated the effects of IL-2 on
coagulation and fibrinolysis in 14 **patients** receiving 12
or 18 times 10⁶ IU/m²/d of IL-2 given as a 15 min infusion for 5 d.
Blood samples were drawn at short intervals after the first IL-2 infusion.
The parameters were analysed by way of analysis for repeated measures (F
tests rather than t tests). During the first day, **thrombin**
-antithrombin (TAT) complexes started to increase 2 h after the IL-2
infusion, reaching peak levels at 4 h (n = 14; 11.2 +/- 6.4 mu-g/l v 49.8
+/- 49.2 mu-g/l, P lt 0.01). Plasmin alpha-2 antiplasmin (PAP) complexes
showed a similar pattern rising from a mean baseline value of 17.5 +/- 7.6
nmol/l to 66.8 +/- 47.7 nmol at 4 h (P lt 0.01). In four **patients**
the peak of PAP preceeded that of TAT. Tissue plasminogen activator (tPA)
rose from a mean baseline value of 4.9 +/- 3.7 mu-g/l to 26.3 +/- 13.5
mu-g/l at 4 h (P lt 0.01). Plasminogen-activator-inhibitor-1 (PAI-1)
levels increased from 59 +/- 35 mu-g/l to 113 +/- 39 mu-g/l at 6 h (P lt
0.01). tPA PAI-1 complexes increased from 0.15 +/- 0.07 to 0.69 +/- 0.21
nmol/l at 6 h (P lt . 0.01). Our study indicates that IL-2 activates the
coagulation and fibrinolytic systems in vivo. The changes resemble
the perturbations observed after endotoxin/**TNF** administration.
These abnormalities may play a role in the side-effects induced by IL-2
therapy.

L25 ANSWER 3 OF 58 MEDLINE on STN DUPLICATE 1
 ACCESSION NUMBER: 96198385 MEDLINE
 DOCUMENT NUMBER: 96198385 PubMed ID: 8609750
 TITLE: Tumour necrosis factor and inducible nitric oxide synthase
 in dilated cardiomyopathy.
 COMMENT: Comment in: Lancet. 1996 Apr 27;347(9009):1129-30
 AUTHOR: Habib F M; Springall D R; Davies G J; Oakley C M; Yacoub M
 H; Polak J M
 CORPORATE SOURCE: Department of Cardiology, Royal Postgraduate Medical
 School, Hammersmith Hospital, London, UK.
 SOURCE: LANCET, (1996 Apr 27) 347 (9009) 1151-5.
 Journal code: 2985213R. ISSN: 0140-6736.
 PUB. COUNTRY: ENGLAND: United Kingdom
 DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
 LANGUAGE: English
 FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
 ENTRY MONTH: 199605
 ENTRY DATE: Entered STN: 19960605
 Last Updated on STN: 19960605
 Entered Medline: 19960529

AB BACKGROUND: Two important features of dilated cardiomyopathy (DCM) are low myocardial contractility and risk of **thromboembolism**. Nitric oxide (NO) exerts a negative inotropic effect on the myocardium and is produced by NO-synthase, an inducible form of which (iNOS) is stimulated by tumour necrosis factor (**TNF-alpha**). Accordingly, we hypothesized that locally produced **TNF-alpha** might contribute to the pathogenesis and complications of DCM by inducing iNOS in the **heart**. METHODS:iNOS and **TNF-alpha** were quantified by histochemistry and computerised image analysis in explanted **heart** tissues or myocardial biopsy material from **patients** with DCM (n = 21) or ischaemic **heart** disease (HD; n = 10) and from normal donor **hearts** (n = 9). FINDINGS: Immunoreactivity for iNOS was strong in myocytes of DCM **hearts**, particularly in areas adjacent to the endocardium, and moderately intense in blood vessels of DCM and IHD **hearts**. The median optical density of the immunostaining for iNOS was greater in **cardiac** myocytes of **patients** with DCM (0.86, range 0.21 to 1.29) than in those from **patients** with IHD (0.20, range 0.095 to 0.26) (p < 0.01) or controls (0.01, range 0.001 to 0.02) (p < 0.001). Staining for **TNF-alpha** was observed in the vascular endothelium and smooth muscle cells of **patients** with DCM but not in IHD or control tissues. INTERPRETATION: The localisation of iNOS and **TNF-alpha** within **cardiac** tissues in DCM suggests that **TNF-alpha** contributes to both the low contractility and the tendency to **thromboembolism** in these **patients**.

L56 ANSWER 18 OF 26 USPATFULL on STN

ACCESSION NUMBER: 95:47750 USPATFULL

TITLE: TNF inhibitors

INVENTOR(S): Christensen, IV, Siegfried B., Philadelphia, PA, United States

Esser, Klaus M., Downingtown, PA, United States

Simon, Philip L., Randolph, NJ, United States

PATENT ASSIGNEE(S): SmithKline Beecham Corp., Philadelphia, PA, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5420154		19950530	<--
	WO 9015534		19901227	<--
APPLICATION INFO.:	US 1992-852180		19920330 (7)	<--
	WO 1991-US5350		19910729	<--
			19920330	PCT 371 date
			19920330	PCT 102(e) date
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1990-562761, filed on 3 Aug 1990, now abandoned			
DOCUMENT TYPE:	Utility			
FILE SEGMENT:	Granted			
PRIMARY EXAMINER:	Springer, David B.			
LEGAL REPRESENTATIVE:	Dinner, Dara L.; Venetianer, Stephen, Lentz, Edward T.			
NUMBER OF CLAIMS:	8			
EXEMPLARY CLAIM:	1			
LINE COUNT:	726			
CAS INDEXING IS AVAILABLE FOR THIS PATENT.				
PI	US 5420154		19950530	<--
	WO 9015534		19901227	<--
AI	US 1992-852180		19920330 (7)	<--
	WO 1991-US5350		19910729	<--
			19920330	PCT 371 date
			19920330	PCT 102(e) date

DETD . . . experimental cerebral malaria (ECM) that reproduces some features of the human disease was prevented in mice by administration of an anti-TNF antibody. [See, Grau et al., Imm. Review 112:49-70 (1989)]. Levels of serum TNF correlated directly with the severity of disease and. . . Diseases. The deposition of silica particles leads to silicosis, a disease of progressive respiratory failure caused by a fibrotic reaction. **Antibody to TNF** completely blocked the silica-induced lung fibrosis in mice [See Piguet et al., Nature, 344:245-247 (1990)]. High levels of TNF production. . . blood flow [See, Vedder et al., PNAS 87:2643-2646 (1990)]. TNF also alters the properties of endothelial cells and has various pro-coagulant activities, such as producing an increase in tissue factor pro-coagulant activity and suppression of the anticoagulant protein C pathway as well as down-regulating the expression of **thrombomodulin** [See, Sherry et al., J. Cell Biol. 107:11269-1277 (1988)]. TNF also has pro-inflammatory activities which together with its early production. . . an inflammatory event) make it a likely mediator of tissue injury in several important disorders including but not limited to, **myocardial infarction**, stroke and circulatory shock. Of specific importance may be TNF-induced expression of adhesion molecules, such as intercellular adhesion molecule (ICAM). . .

L80 ANSWER 7 OF 12

MEDLINE on STN

DUPLICATE 1

ACCESSION NUMBER: 95202670 MEDLINE

DOCUMENT NUMBER: 95202670 PubMed ID: 7534645

TITLE: Neutrophil activation in paediatric extracorporeal circuits: effect of circulation and temperature variation.

AUTHOR: el Habbal M H; Carter H; Smith L J; Elliott M J; Strobel S

CORPORATE SOURCE: Cardiothoracic Unit, Hospital for Sick Children, London, United Kingdom.

SOURCE: CARDIOVASCULAR RESEARCH, (1995 Jan) 29 (1) 102-7.

Journal code: 0077427. ISSN: 0008-6363.

PUB. COUNTRY: ENGLAND: United Kingdom

DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)

LANGUAGE: English

FILE SEGMENT: Priority Journals

ENTRY MONTH: 199504

ENTRY DATE: Entered STN: 19950504

Last Updated on STN: 20000303

Entered Medline: 19950421

AB OBJECTIVE: Upregulation of neutrophil adhesion molecules (CD11b and L-selectin) and release of a modulating cytokine (IL8) have been reported in vivo and in vitro in adult cardiopulmonary bypass. The aim of this study was to determine whether paediatric bypass preparations have similar influences and whether neutrophil-endothelium interactions are required for IL8 release. METHODS: In vitro paediatric cardiopulmonary bypass circuits (n = 15) were constructed (identical to those used clinically), as well as static loops (n = 15) using donor blood. The effects of circulation and temperature (17 degrees C, 25 degrees C, 37 degrees C) on the initiation of acute inflammation were examined. Cellular expressions of neutrophil adhesion molecules CD11b and L-selectin were assayed by immunofluorescence technique, and serum IL8, IL6, **TNF**-alpha, leucocyte elastase, and terminal complement complex were measured by ELISA. RESULTS: In all experiments, an immediate increase in CD11b expression occurred [median values, in relative fluorescence units: 64.9 (range 45.3-212.9) at rest; 365.2 (205-835.4) at 10 min; P < 0.001], along with a decrease in L-selectin expression [153.5 (115.5-220.7) at rest; 42 (12-134) at 10 min; P < 0.01]. Serum concentrations of the following increased gradually and were higher in circulation than in static loops: IL8 [1500 (500-2500) pg.ml⁻¹ in circuit v 600 (180-1500) pg.ml⁻¹ in loop, P < 0.001]; **TNF**-alpha P < 0.05]; and terminal complement complex [25.9 (6.8-120) v 4.7 (0-21.6) AU.ml⁻¹, P < 0.01]. Cooling decreased and rewarming increased upregulation of CD11b and downregulation of L-selectin and release of IL8. IL6 was undetectable. CONCLUSIONS: In the absence of endothelium, in vitro paediatric cardiopulmonary bypass causes profound acute inflammatory changes in donor blood with release of IL8. These changes were greater than in adult cardiopulmonary bypass. Temperature variation and circulation modulate the responses.

L11. ANSWER 2 OF 4 MEDLINE
 ACCESSION NUMBER: 95393687 MEDLINE
 DOCUMENT NUMBER: 95393687 PubMed ID: 7664546
 TITLE: CDP571, a humanized antibody to human tumor necrosis factor-alpha: safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock. CPD571 Sepsis Study Group.
 AUTHOR: Dhainaut J F; Vincent J L; Richard C; Lejeune P; Martin C; Fierobe L; Stephens S; Ney U M; Sopwith M
 CORPORATE SOURCE: Intensive Care Units, Cochin Port-Royal University Hospital, Paris, France.
 SOURCE: CRITICAL CARE MEDICINE, (1995 Sep) 23 (9) 1461-9. Journal code: DTF; 0355501. ISSN: 0090-3493.
 PUB. COUNTRY: United States
 (CLINICAL TRIAL)
 (CLINICAL TRIAL, PHASE II)
 Journal; Article; (JOURNAL ARTICLE)
 (MULTICENTER STUDY)
 (RANDOMIZED CONTROLLED TRIAL)
 LANGUAGE: English
 FILE SEGMENT: Abridged Index Medicus Journals; Priority Journals
 ENTRY MONTH: 199510
 ENTRY DATE: Entered STN: 19951020
 Last Updated on STN: 19951020
 Entered Medline: 19951011
 AB OBJECTIVES: To determine the safety of a "humanized" antibody to human **anti-tumor necrosis factor** -alpha (TNF-alpha) in patients with septic shock, and to examine the pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in this patient group. DESIGN: Prospective, randomized, placebo-controlled, phase II multicenter clinical trial, with escalating doses of a fully **humanized anti-TNF** -alpha antibody (CDP571). SETTING: Seven academic intensive care units in Europe. PATIENTS: Forty-two patients with rapidly evolving septic shock who received CDP571 in addition to standard supportive care. INTERVENTIONS: Patients received intravenously either placebo or one of four single doses of CDP571: 0.1, 0.3, 1.0, or 3.0 mg/kg. MEASUREMENTS AND MAIN RESULTS: The **humanized anti-TNF-alpha** antibody was well tolerated. The overall all-cause 28-day mortality rate was 62%. Mortality rate was similar in the placebo and treatment groups, except that all six patients who received 0.3 mg/kg of CDP571 died within 7 days. This outcome, which was not dose-related, is consistent with the poorer prognostic characteristics of this group at baseline. The peak CDP571 concentrations and area under the curve increased proportionately with the dose. The low level of the immune response detected had little effect on the ability of circulating CDP571 to bind TNF-alpha and on the pharmacokinetics of the antibody. An abrupt reduction in circulating TNF-alpha concentration was observed 30 mins after CDP571 administration at all active dosage levels. While interleukin-1 beta and interleukin-6 plasma concentrations decreased with time in all dosage groups, these cytokine concentrations decreased more rapidly during the initial 24 hrs in the treatment groups than in the placebo group. CONCLUSIONS: The **humanized anti-TNF-alpha** antibody, CDP571, is well tolerated and able to cause a dose-dependent reduction in circulating

TNF-alpha concentrations in patients with septic shock. Further studies are needed to determine the efficacy of this antibody to improve the survival rates of critically ill patients with severe sepsis.

L11 ANSWER 3 OF 4 MEDLINE

(FILE 'USPAT' ENTERED AT 16:39:17 ON 12 FEB 97)

L1 1473 S TNF
L2 1202 S TUMOR NECROSIS FACTOR
L3 8092 S ANTAGONISTS
L4 13046 S ANTAGONIST?
L5 80 S L4 (15A) (L1 OR L2)
L6 22123 S ANTIBOD?
L7 238 S L6 (15A) (L1 OR L2)
L8 7381 S MYOCARDI?
L9 1114 S THROMBOLYTIC
L10 10043 S CARDIOVASCULAR
L11 1527 S THROMBOTIC
L12 1215 S CEREBROVASCULAR
L13 16877 S L8 OR L9 OR L10 OR L10 OR L11 OR L12
L14 49 S L13 AND L7
L15 44 S L13 AND L5
L16 85 S L14 OR L15
L17 340629 S TREATMENT
L18 780007 S PREVENT
L19 49 S L14 AND L17
L20 20 S L15 AND L17

=>

**(19) World Intellectual Property Organization
International Bureau**



(43) International Publication Date
13 December 2001 (13.12.2001)

PCT

(10) International Publication Number
WO 01/94585 A1

(51) International Patent Classification⁷: C12N 15/13,
C07K 16/24, 16/46, A61K 47/48, C07K 19/00, C12N
15/62, 15/70, 1/21, A61K 39/395, A61P 19/02, 37/06

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(21) International Application Number: PCT/GB01/02477

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(22) International Filing Date: 5 June 2001 (05.06.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0013810.7 6 June 2000 (06.06.2000) GB

(81) Designated States (national): AE, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

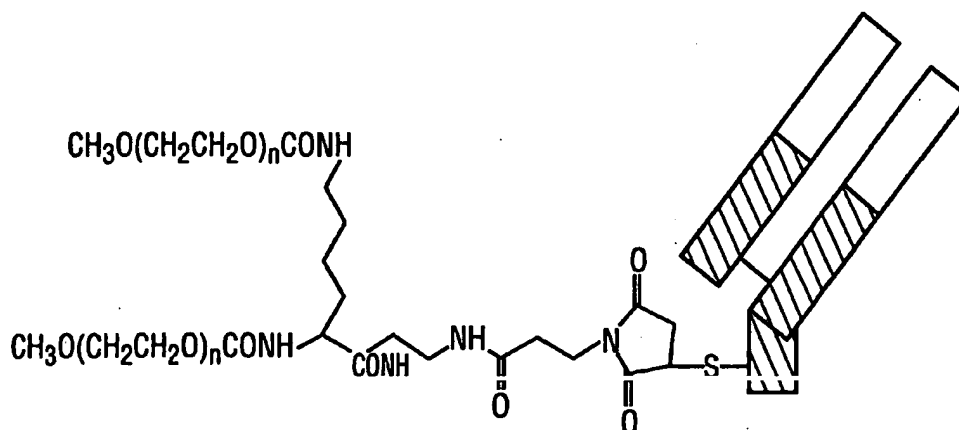
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Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTIBODY MOLECULES HAVING SPECIFICITY FOR HUMAN TUMOR NECROSIS FACTOR ALPHA, AND USE THEREOF



(57) Abstract: There is disclosed antibody molecules containing at least one CDR derived from a mouse monoclonal antibody having specificity for human TNF α . There is also disclosed a CDR grafted antibody wherein at least one of the CDRs is a hybrid CDR. Further disclosed are DNA sequences encoding the chains of the antibody molecules, vectors, transformed host cells and uses of the antibody molecules in the treatment of diseases mediated by TNF α .

WO 01/94,585 A1

ANTIBODY MOLECULES HAVING SPECIFICITY FOR HUMAN TUMOR NECROSIS FACTOR ALPHA, AND USE THEREOF

The present invention relates to an antibody molecule having specificity for antigenic determinants of human tumour necrosis factor alpha (TNF α). The present invention also relates to the therapeutic uses of the antibody molecule and methods for producing the antibody molecule.

This invention relates to antibody molecules. In an antibody molecule, there are two heavy chains and two light chains. Each heavy chain and each light chain has at its N-terminal end a variable domain. Each variable domain is composed of four framework regions (FRs) alternating with three complementarily determining regions (CDRs). The residues in the variable domains are conventionally numbered according to a system devised by Kabat *et al.* This system is set forth in Kabat *et al.*, 1987, in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services, NIH, USA (hereafter "Kabat *et al. (supra)*"). This numbering system is used in the present specification except where otherwise indicated.

The Kabat residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. The correct Kabat numbering of residues may be determined for a given antibody by alignment of residues of homology in the sequence of the antibody with a "standard" Kabat numbered sequence.

The CDRs of the heavy chain variable domain are located at residues 31-35 (CDRH1), residues 50-65 (CDRH2) and residues 95-102 (CDRH3) according to the Kabat numbering.

The CDRs of the light chain variable domain are located at residues 24-34 (CDRL1), residues 50-56 (CDRL2) and residues 89-97 (CDRL3) according to the Kabat numbering.

Construction of CDR-grafted antibodies is described in European Patent Application EP-A-0239400, which discloses a process in which the CDRs of a mouse monoclonal antibody are grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The

CDRs determine the antigen binding specificity of antibodies and are relatively short peptide sequences carried on the framework regions of the variable domains.

The earliest work on humanising monoclonal antibodies by CDR-grafting was carried out on monoclonal antibodies recognising synthetic antigens, such as NP. However, examples in which a mouse monoclonal antibody recognising lysozyme and a rat monoclonal antibody recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen *et al.* (Science, 239, 1534-1536, 1988) and Riechmann *et al.* (Nature, 332, 323-324, 1988), respectively.

Riechmann *et al.*, found that the transfer of the CDRs alone (as defined by Kabat (Kabat *et al.* (*supra*) and Wu *et al.*, J. Exp. Med., 132, 211-250, 1970)) was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. It was found that a number of framework residues have to be altered so that they correspond to those of the donor framework region. Proposed criteria for selecting which framework residues need to be altered are described in International Patent Application WO 90/07861.

A number of reviews discussing CDR-grafted antibodies have been published, including Vaughan *et al.* (Nature Biotechnology, 16, 535-539, 1998).

TNF α is a pro-inflammatory cytokine that is released by and interacts with cells of the immune system. Thus, TNF α is released by macrophages that have been activated by lipopolysaccharides (LPS) of gram negative bacteria. As such, TNF α appears to be an endogenous mediator of central importance involved in the development and pathogenesis of endotoxic shock associated with bacterial sepsis. TNF α has also been shown to be up-regulated in a number of human diseases, including chronic diseases such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis. Mice transgenic for human TNF α produce high levels of TNF α constitutively and develop a spontaneous, destructive polyarthritis resembling rheumatoid arthritis (Kaffer *et al.*, EMBO J., 10, 4025-4031, 1991). TNF α is therefore referred to as a pro-inflammatory cytokine.

Monoclonal antibodies against TNF α have been described in the prior art. Meager *et al.*, (Hybridoma, 6, 305-311, 1987) describe murine monoclonal antibodies against recombinant TNF α . Fendly *et al.*, (Hybridoma, 6, 359-370, 1987) describe the use of murine monoclonal antibodies against recombinant TNF α in defining neutralising epitopes on TNF. Shimamoto *et al.*, (Immunology Letters, 17, 311-318, 1988) describe the use of murine monoclonal antibodies against TNF γ and their use in preventing endotoxic shock in mice. Furthermore, in International Patent Application WO 92/11383, recombinant

antibodies, including CDR-grafted antibodies, specific for TNF α are disclosed. Rankin *et al.*, (British J. Rheumatology, 34, 334-342, 1995) describe the use of such CDR-grafted antibodies in the treatment of rheumatoid arthritis. US-A-5 919 452 discloses anti-TNF chimeric antibodies and their use in treating pathologies associated with the presence of
5 TNF.

Antibodies to TNF α have been proposed for the prophylaxis and treatment of endotoxic shock (Beutler *et al.*, Science, 234, 470-474, 1985). Bodmer *et al.*, (Critical Care Medicine, 21, S441-S446, 1993) and Wherry *et al.*, (Critical Care Medicine, 21, S436-S440, 1993) discuss the therapeutic potential of anti-TNF α antibodies in the treatment of
10 septic shock. The use of anti-TNF α antibodies in the treatment of septic shock is also discussed by Kirschenbaum *et al.*, (Critical Care Medicine, 26, 1625-1626, 1998). Collagen-induced arthritis can be treated effectively using an anti-TNF α monoclonal antibody (Williams *et al.* (PNAS-USA, 89, 9784-9788, 1992)).

Increased levels of TNF α are found in both the synovial fluid and peripheral blood
15 of patients suffering from rheumatoid arthritis. When TNF α blocking agents are administered to patients suffering from rheumatoid arthritis, they reduce inflammation, improve symptoms and retard joint damage (McKown *et al.* (Arthritis Rheum., 42, 1204-1208, 1999).

The use of anti-TNF α antibodies in the treatment of rheumatoid arthritis and
20 Crohn's disease is discussed in Feldman *et al.*, (Transplantation Proceedings, 30, 4126-4127, 1998), Adorini *et al.*, (Trends in Immunology Today, 18, 209-211, 1997) and in Feldman *et al.*, (Advances in Immunology, 64, 283-350, 1997). The antibodies to TNF α used in such treatments are generally chimeric antibodies, such as those described in US-A-5 919 452.

25 Two TNF α blocking products are currently licensed for the treatment of rheumatoid arthritis. The first, called etanercept, is marketed by Immunex Corporation as Enbrel[™]. It is a recombinant fusion protein comprising two p75 soluble TNF-receptor domains linked to the Fc portion of a human immunoglobulin. The second, called infliximab, is marketed by Centocor Corporation as Remicade[™]. It is a chimeric antibody having murine anti-
30 TNF α variable domains and human IgG1 constant domains.

The prior art recombinant anti-TNF α antibody molecules generally have a reduced affinity for TNF α compared to the antibodies from which the variable regions or CDRs are

derived, generally have to be produced in mammalian cells and are expensive to manufacture. Prior art anti-TNF α antibodies are described in Stephens *et al.*, (Immunology, 85; 668-674, 1995), GB-A-2 246 570 and GB-A-2 297 145.

There is a need for an antibody molecule to treat chronic inflammatory diseases
5 which can be used repeatedly and produced easily and efficiently. There is also a need for an antibody molecule which has high affinity for TNF α and low immunogenicity in humans.

In a first aspect, the present invention provides an antibody molecule having specificity for TNF α , comprising a heavy chain wherein the variable domain comprises a
10 CDR (as defined by Kabat *et al.*, (*supra*)) having the sequence given as H1 in Figure 3 (SEQ ID NO:1) for CDRH1, as H2' in Figure 3 (SEQ ID NO:2) or as H2 in Figure 3 (SEQ ID NO:7) for CDRH2 or as H3 in Figure 3 (SEQ ID NO:3) for CDRH3.

The antibody molecule of the first aspect of the present invention comprises at least one CDR selected from H1, H2' or H2 and H3 (SEQ ID NO:1; SEQ ID NO:2 or SEQ ID
15 NO:7 and SEQ ID NO:3) for the heavy chain variable domain. Preferably, the antibody molecule comprises at least two and more preferably all three CDRs in the heavy chain variable domain.

In a second aspect of the present invention, there is provided an antibody molecule having specificity for human TNF α , comprising a light chain wherein the variable domain
20 comprises a CDR (as defined by Kabat *et al.*, (*supra*)) having the sequence given as L1 in Figure 3 (SEQ ID NO:4) for CDRL1, L2 in Figure 3 (SEQ ID NO:5) for CDRL2 or L3 in Figure 3 (SEQ ID NO:6) for CDRL3.

The antibody molecule of the second aspect of the present invention comprises at least one CDR selected from L1, L2 and L3 (SEQ ID NO:4 to SEQ ID NO:6) for the light
25 chain variable domain. Preferably, the antibody molecule comprises at least two and more preferably all three CDRs in the light chain variable domain.

The antibody molecules of the first and second aspects of the present invention preferably have a complementary light chain or a complementary heavy chain, respectively.

Preferably, the antibody molecule of the first or second aspect of the present
30 invention comprises a heavy chain wherein the variable domain comprises a CDR (as defined by Kabat *et al.*, (*supra*)) having the sequence given as H1 in Figure 3 (SEQ ID NO:1) for CDRH1, as H2' or H2 in Figure 3 (SEQ ID NO:2 or SEQ ID NO:7) for CDRH2 or as H3 in Figure 3 (SEQ ID NO:3) for CDRH3 and a light chain wherein the variable

domain comprises a CDR (as defined by Kabat *et al.*, (*supra*)) having the sequence given as L1 in Figure 3 (SEQ ID NO:4) for CDRL1, as L2 in Figure 3 (SEQ ID NO:5) for CDRL2 or as L3 in Figure 3 (SEQ ID NO:6) for CDRL3.

The CDRs given in SEQ IDS NOS:1 and 3 to 7 and in Figure 3 referred to above
5 are derived from a mouse monoclonal antibody hTNF40. However, SEQ ID NO:2 consists of a hybrid CDR. The hybrid CDR comprises part of heavy chain CDR2 from mouse monoclonal antibody hTNF40 (SEQ ID NO:7) and part of heavy chain CDR2 from a human group 3 germline V region sequence.

The complete sequences of the variable domains of the mouse hTNF40 antibody
10 are shown in Figures 6 (light chain) (SEQ ID NO:99) and Figure 7 (heavy chain) (SEQ ID NO:100). This mouse antibody is referred to below as "the donor antibody".

A first alternatively preferred embodiment of the first or second aspect of the present invention is the mouse monoclonal antibody hTNF40 having the light and heavy chain variable domain sequences shown in Figure 6 (SEQ ID NO:99) and Figure 7 (SEQ
15 ID NO:100), respectively. The light chain constant region of hTNF40 is kappa and the heavy chain constant region is IgG2a.

In a second alternatively preferred embodiment, the antibody according to either of the first and second aspects of the present invention is a chimeric mouse/human antibody molecule, referred to herein as the chimeric hTNF40 antibody molecule. The chimeric
20 antibody molecule comprises the variable domains of the mouse monoclonal antibody hTNF40 (SEQ ID NOS:99 and 100) and human constant domains. Preferably, the chimeric hTNF40 antibody molecule comprises the human C kappa domain (Hieter *et al.*, Cell, 22, 197-207, 1980; Genbank accession number J00241) in the light chain and the human gamma 4 domains (Flanagan *et al.*, Nature, 300, 709-713, 1982) in the heavy chain.

In a third alternatively preferred embodiment, the antibody according to either of the first and second aspects of the present invention is a CDR-grafted antibody molecule. The term "a CDR-grafted antibody molecule" as used herein refers to an antibody molecule wherein the heavy and/or light chain contains one or more CDRs (including, if desired, a hybrid CDR) from the donor antibody (e.g. a murine monoclonal antibody) grafted into a
25 heavy and/or light chain variable region framework of an acceptor antibody (e.g. a human antibody).

Preferably, such a CDR-grafted antibody has a variable domain comprising human acceptor framework regions as well as one or more of the donor CDRs referred to above.

When the CDRs are grafted, any appropriate acceptor variable region framework sequence may be used having regard to the class/type of the donor antibody from which the CDRs are derived, including mouse, primate and human framework regions. Examples of human frameworks which can be used in the present invention are KOL, NEWM, REI, EU, 5 TUR, TEL, LAY and POM (Kabat *et al. (supra)*). For example, KOL and NEWM can be used for the heavy chain, REI can be used for the light chain and EU, LAY and POM can be used for both the heavy chain and the light chain. The preferred framework regions for the light chain are the human group 1 framework regions shown in Figure 1 (SEQ ID NOS:83, 85, 87 and 89). The preferred framework regions for the heavy chain are the 10 human group 1 and group 3 framework regions shown in Figure 2 (SEQ ID NOS:91, 93, 95 and 97 and SEQ ID NOS:106, 107, 108 and 109), respectively.

In a CDR-grafted antibody of the present invention, it is preferred to use as the acceptor antibody one having chains which are homologous to the chains of the donor antibody. The acceptor heavy and light chains do not necessarily need to be derived from 15 the same antibody and may, if desired, comprise composite chains having framework regions derived from different chains.

Also, in a CDR-grafted antibody of the present invention, the framework regions need not have exactly the same sequence as those of the acceptor antibody. For instance, unusual residues may be changed to more frequently-occurring residues for that acceptor 20 chain class or type. Alternatively, selected residues in the acceptor framework regions may be changed so that they correspond to the residue found at the same position in the donor antibody. Such changes should be kept to the minimum necessary to recover the affinity of the donor antibody. A protocol for selecting residues in the acceptor framework regions which may need to be changed is set forth in WO 91/09967.

25 Preferably, in a CDR-grafted antibody molecule of the present invention, if the acceptor heavy chain has human group 1 framework regions (shown in Figure 2) (SEQ ID NOS:91, 93, 95 and 97), then the acceptor framework regions of the heavy chain comprise, in addition to one or more donor CDRs, donor residues at positions 28, 69 and 71 (according to Kabat *et al. (supra)*).

30 Alternatively, if the acceptor heavy chain has group 1 framework regions, then the acceptor framework regions of the heavy chain comprise, in addition to one or more donor CDRs, donor residues at positions 28, 38, 46, 67, 69 and 71 (according to Kabat *et al. (supra)*).

Preferably, in a CDR-grafted antibody molecule of the present invention, if the acceptor heavy chain has human group 3 framework regions (shown in Figure 2) (SEQ ID NOS:106, 107, 108 and 109), then the acceptor framework regions of the heavy chain comprise, in addition to one or more donor CDRs, donor residues at positions 27, 28, 30, 48, 49, 69, 71, 73, 76 and 78 (according to Kabat *et al. (supra)*).

Preferably, in a CDR-grafted antibody molecule according to the present invention, if the acceptor light chain has human group 1 framework regions (shown in Figure 1) (SEQ ID NOS:83, 85, 87 and 89) then the acceptor framework regions of the light chain comprise donor residues at positions 46 and 60 (according to Kabat *et al. (supra)*).

Donor residues are residues from the donor antibody, i.e. the antibody from which the CDRs were originally derived.

The antibody molecule of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, modified Fab, Fab', F(ab')₂ or Fv fragment; a light chain or heavy chain monomer or dimer; a single chain antibody, e.g. a single chain Fv in which the heavy and light chain variable domains are joined by a peptide linker. Similarly, the heavy and light chain variable regions may be combined with other antibody domains as appropriate.

Preferably the antibody molecule of the present invention is a Fab fragment. Preferably the Fab fragment has a heavy chain having the sequence given as SEQ ID NO:111 and a light chain having the sequence given as SEQ ID NO:113. The amino acid sequences given in SEQ ID NO:111 and SEQ ID NO:113 are preferably encoded by the nucleotide sequences given in SEQ ID NO:110 and SEQ ID NO:112, respectively.

Alternatively, it is preferred that the antibody molecule of the present invention is a modified Fab fragment wherein the modification is the addition to the C-terminal end of its heavy chain one or more amino acids to allow the attachment of an effector or reporter molecule. Preferably, the additional amino acids form a modified hinge region containing one or two cysteine residue to which the effector or reporter molecule may be attached. Such a modified Fab fragment preferably has a heavy chain having the sequence given as SEQ ID NO:115 and the light chain having the sequence given as SEQ ID NO:113. The amino acid sequence given in SEQ ID NO:115 is preferably encoded by the nucleotide sequence given in SEQ ID NO:114.

A preferred effector group is a polymer molecule, which may be attached to the modified Fab fragment to increase its half-life *in vivo*.

The polymer molecule may, in general, be a synthetic or a naturally occurring polymer, for example an optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide, e.g. a homo- or hetero- polysaccharide.

5 Particular optional substituents which may be present on the above-mentioned synthetic polymers include one or more hydroxy, methyl or methoxy groups. Particular examples of synthetic polymers include optionally substituted straight or branched chain poly(ethyleneglycol), poly(propyleneglycol) poly(vinylalcohol) or derivatives thereof, especially optionally substituted poly(ethyleneglycol) such as methoxypoly(ethyleneglycol)
10 or derivatives thereof. Particular naturally occurring polymers include lactose, amylose, dextran, glycogen or derivatives thereof. "Derivatives" as used herein is intended to include reactive derivatives, for example thiol-selective reactive groups such as maleimides and the like. The reactive group may be linked directly or through a linker segment to the polymer. It will be appreciated that the residue of such a group will in some instances form
15 part of the product as the linking group between the antibody fragment and the polymer.

The size of the polymer may be varied as desired, but will generally be in an average molecular weight range from 500Da to 50000Da, preferably from 5000 to 4000Da and more preferably from 25000 to 4000Da. The polymer size may in particular be selected on the basis of the intended use of the product. Thus, for example, where the
20 product is intended to leave the circulation and penetrate tissue, for example for use in the treatment of a tumour, it may be advantageous to use a small molecular weight polymer, for example with a molecular weight of around 5000Da. For applications where the product remains in the circulation, it may be advantageous to use a higher molecular weight polymer, for example having a molecular weight in the range from 25000Da to 40000Da.

25 Particularly preferred polymers include a polyalkylene polymer, such as a poly(ethyleneglycol) or, especially, a methoxypoly(ethyleneglycol) or a derivative thereof, and especially with a molecular weight in the range from about 25000Da to about 40000Da.

Each polymer molecule attached to the modified antibody fragment may be
30 covalently linked to the sulphur atom of a cysteine residue located in the fragment. The covalent linkage will generally be a disulphide bond or, in particular, a sulphur-carbon bond.

Where desired, the antibody fragment may have one or more effector or reporter molecules attached to it. The effector or reporter molecules may be attached to the antibody fragment through any available amino acid side-chain or terminal amino acid functional group located in the fragment, for example any free amino, imino, hydroxyl or
5 carboxyl group.

An activated polymer may be used as the starting material in the preparation of polymer-modified antibody fragments as described above. The activated polymer may be any polymer containing a thiol reactive group such as an α -halocarboxylic acid or ester, e.g. iodoacetamide, an imide, e.g. maleimide, a vinyl sulphone or a disulphide. Such
10 starting materials may be obtained commercially (for example from Shearwater Polymers Inc., Huntsville, AL, USA) or may be prepared from commercially available starting materials using conventional chemical procedures.

As regards attaching poly(ethyleneglycol) (PEG) moieties, reference is made to "Poly(ethyleneglycol) Chemistry, Biotechnical and Biomedical Applications", 1992, J.
15 Milton Harris (ed), Plenum Press, New York, "Poly(ethyleneglycol) Chemistry and Biological Applications", 1997, J. Milton Harris and S. Zalipsky (eds), American Chemical Society, Washington DC and "Bioconjugation Protein Coupling Techniques for the Biomedical Sciences", 1998, M. Aslam and A. Dent, Grove Publishers, New York.

Where it is desired to obtain an antibody fragment linked to an effector or reporter
20 molecule, this may be prepared by standard chemical or recombinant DNA procedures in which the antibody fragment is linked either directly or via a coupling agent to the effector or reporter molecule either before or after reaction with the activated polymer as appropriate. Particular chemical procedures include, for example, those described in WO 93/62331, WO 92/22583, WO 90,195 and WO 89/1476. Alternatively, where the effector
25 or reporter molecule is a protein or polypeptide the linkage may be achieved using recombinant DNA procedures, for example as described in WO 86/01533 and EP-A-0392745.

Preferably, the modified Fab fragment of the present invention is PEGylated (i.e. has PEG (poly(ethyleneglycol)) covalently attached thereto) according to the method
30 disclosed in EP-A-0948544. Preferably the antibody molecule of the present invention is a PEGylated modified Fab fragment as shown in Figure 13. As shown in Figure 13, the modified Fab fragment has a maleimide group covalently linked to a single thiol group in a modified hinge region. A lysine residue is covalently linked to the maleimide group. To

each of the amine groups on the lysine residue is attached a methoxypoly(ethyleneglycol) polymer having a molecular weight of approximately 20,000 Da. The total molecular weight of the entire effector molecule is therefore approximately 40,000 Da.

Preferably, in the compound shown in Figure 13, the heavy chain of the antibody
5 part has the sequence given as SEQ ID NO:115 and the light chain has the sequence given in SEQ ID NO:113. This compound is referred to herein as CDP870.

The constant region domains of the antibody molecule of the present invention, if present, may be selected having regard to the proposed function of the antibody molecule, and in particular the effector functions which may be required. For example, the constant
10 region domains may be human IgA, IgD, IgE, IgG or IgM domains. In particular, human IgG constant region domains may be used, especially of the IgG1 and IgG3 isotypes when the antibody molecule is intended for therapeutic uses and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g.
15 for simply blocking TNF α activity.

Also, the antibody molecule of the present invention may have an effector or a reporter molecule attached to it. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, procedures of recombinant DNA technology may be used to produce an
20 antibody molecule in which the Fc fragment (CH2, CH3 and hinge domains), the CH2 and CH3 domains or the CH3 domain of a complete immunoglobulin molecule has (have) been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

The antibody molecule of the present invention preferably has a binding affinity of
25 at least 0.85×10^{-10} M, more preferably at least 0.75×10^{-10} M and most preferably at least 0.5×10^{-10} M. (It is worth noting that the preferred humanised antibody molecule of the present invention, as described below, has an affinity of about 0.5×10^{-10} M, which is better than the affinity of the murine monoclonal antibody from which it is derived. The murine antibody has an affinity of about 0.85×10^{-10} M.)

30 Preferably, the antibody molecule of the present invention comprises the light chain variable domain hTNF40-gL1 (SEQ ID NO:8) and the heavy chain variable domain gh3hTNF40.4 (SEQ ID NO:11). The sequences of the variable domains of these light and heavy chains are shown in Figures 8 and 11, respectively.

The present invention also relates to variants of the antibody molecule of the present invention, which have an improved affinity for TNF α . Such variants can be obtained by a number of affinity maturation protocols including mutating the CDRs (Yang *et al.*, J. Mol. Biol., 254, 392-403, 1995), chain shuffling (Marks *et al.*, Bio/Technology, 10, 779-783, 5 1992), use of mutator strains of *E. coli* (Low *et al.*, J. Mol. Biol., 250, 359-368, 1996), DNA shuffling (Patten *et al.*, Curr. Opin. Biotechnol., 8, 724-733, 1997), phage display (Thompson *et al.*, J. Mol. Biol., 256, 77-88, 1996) and sexual PCR (Cramer *et al.*, Nature, 391, 288-291, 1998). Vaughan *et al.* (*supra*) discusses these methods of affinity maturation.

10 The present invention also provides a DNA sequence encoding the heavy and/or light chain(s) of the antibody molecule of the present invention.

Preferably, the DNA sequence encodes the heavy or the light chain of the antibody molecule of the present invention.

In one preferred embodiment, the DNA sequence encodes a light chain and 15 comprises the sequence shown in SEQ ID NO:8 (hTNF40-gL1) or SEQ ID NO:9 (h-TNF-40-gL2) or a degenerate equivalent thereof.

In an alternatively preferred embodiment, the DNA sequence encodes a heavy chain and comprises the sequence shown in SEQ ID NO:10 (gh1hTNF40.4) or SEQ ID NO:11 (gh3hTNF40.4) or a degenerate equivalent thereof.

20 The DNA sequence of the present invention may comprise synthetic DNA, for instance produced by chemical processing, cDNA, genomic DNA or any combination thereof.

The present invention also relates to a cloning or expression vector comprising one or more DNA sequences of the present invention. Preferably, the cloning or expression 25 vector comprises two DNA sequences, encoding the light chain and the heavy chain of the antibody molecule of the present invention, respectively.

In a preferred embodiment, the present invention provides an *E. coli* expression vector comprising a DNA sequence of the present invention. Preferably the expression vector is pTTO(CDP870) as shown schematically in Figure 22.

30 The present invention also comprises vector pDNA_{Ab}Eng-G1 as shown in Figure 19.

General methods by which the vectors may be constructed, transfection methods and culture methods are well known to those skilled in the art. In this respect, reference is made to "Current Protocols in Molecular Biology", 1999, F. M. Ausubel (ed), Wiley

Interscience, New York and the Maniatis Manual produced by Cold Spring Harbor Publishing.

DNA sequences which encode the antibody molecule of the present invention can be obtained by methods well known to those skilled in the art. For example, DNA
5 sequences coding for part or all of the antibody heavy and light chains may be synthesised as desired from the determined DNA sequences or on the basis of the corresponding amino acid sequences.

DNA coding for acceptor framework sequences is widely available to those skilled in the art and can be readily synthesised on the basis of their known amino acid sequences.

10 Standard techniques of molecular biology may be used to prepare DNA sequences coding for the antibody molecule of the present invention. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate.

Any suitable host cell/vector system may be used for expression of the DNA
15 sequences encoding the antibody molecule of the present invention. Bacterial, for example *E. coli*, and other microbial systems may be used, in part, for expression of antibody fragments such as Fab and F(ab')₂ fragments, and especially Fv fragments and single chain antibody fragments, for example, single chain Fvs. Eukaryotic, e.g. mammalian, host cell expression systems may be used for production of larger antibody molecules, including
20 complete antibody molecules. Suitable mammalian host cells include CHO, myeloma or hybridoma cells.

The present invention also provides a process for the production of an antibody molecule according to the present invention comprising culturing a host cell comprising a vector of the present invention under conditions suitable for leading to expression of
25 protein from DNA encoding the antibody molecule of the present invention, and isolating the antibody molecule.

Preferably the process for the production of the antibody molecule of the present invention comprises culturing *E. coli* comprising an *E. coli* expression vector comprising the DNA sequence of the present invention under conditions suitable for leading to
30 expression of protein from the DNA sequence and isolating the antibody molecule. The antibody molecule may be secreted from the cell or targeted to the periplasm by suitable signal sequences. Alternatively, the antibody molecules may accumulate within the cell's cytoplasm. Preferably the antibody molecule is targeted to the periplasm. Depending on

the antibody molecule being produced and the process used, it is desirable to allow the antibody molecules to refold and adopt a functional conformation. Procedures for allowing antibody molecules to refold are well known to those skilled in the art.

The antibody molecule may comprise only a heavy or light chain polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence needs to be used to transfect the host cells. For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, a first vector encoding a light chain polypeptide and a second vector encoding a heavy chain polypeptide. Alternatively, a single vector may be used, the vector including sequences encoding light chain and heavy chain polypeptides.

The present invention also provides a therapeutic or diagnostic composition comprising an antibody molecule of the present invention in combination with a pharmaceutically acceptable excipient, diluent or carrier.

The present invention also provides a process for preparation of a therapeutic or diagnostic composition comprising admixing the antibody molecule of the present invention together with a pharmaceutically acceptable excipient, diluent or carrier.

The antibody molecule may be the sole active ingredient in the therapeutic or diagnostic composition or may be accompanied by other active ingredients including other antibody ingredients, for example anti-T cell, anti-IFN γ or anti-LPS antibodies, or non-antibody ingredients such as xanthines.

The pharmaceutical compositions should preferably comprise a therapeutically effective amount of the antibody of the invention. The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent needed to treat, ameliorate or prevent a targeted disease or condition, or to exhibit a detectable therapeutic or preventative effect. For any antibody, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually in rodents, rabbits, dogs, pigs or primates. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

The precise effective amount for a human subject will depend upon the severity of the disease state, the general health of the subject, the age, weight and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities and tolerance/response to therapy. This amount can be determined by routine experimentation

and is within the judgement of the clinician. Generally, an effective dose will be from 0.01 mg/kg to 50 mg/kg, preferably 0.1 mg/kg to 20 mg/kg, more preferably about 15 mg/kg. As shown in the Examples below, doses of 1, 5 and 20 mg/kg have been used to treat patients suffering from rheumatoid arthritis.

- 5 Compositions may be administered individually to a patient or may be administered in combination with other agents, drugs or hormones.

The dose at which the antibody molecule of the present invention is administered depends on the nature of the condition to be treated, the degree to which the level of TNF α to be neutralised is, or is expected to be, raised above a desirable level, and on whether the
10 antibody molecule is being used prophylactically or to treat an existing condition.

Thus, for example, where the product is for treatment or prophylaxis of a chronic inflammatory disease, such as rheumatoid arthritis, suitable doses of the antibody molecule of the present invention lie in the range of between 0.5 and 50 mg/kg, more preferably between 1 and 20 mg/kg and most preferably about 15 mg/kg. The frequency of dose will
15 depend on the half-life of the antibody molecule and the duration of its effect.

If the antibody molecule has a short half-life (e.g. 2 to 10 hours) it may be necessary to give one or more doses per day. Alternatively, if the antibody molecule has a long half life (e.g. 2 to 15 days) it may only be necessary to give a dosage once per day, per week or even once every 1 or 2 months.

- 20 A pharmaceutical composition may also contain a pharmaceutically acceptable carrier for administration of the antibody. The carrier should not itself induce the production of antibodies harmful to the individual receiving the composition and should not be toxic. Suitable carriers may be large, slowly metabolised macromolecules such as proteins, polypeptides, liposomes, polysaccharides, polylactic acids, polyglycolic acids,
25 polymeric amino acids, amino acid copolymers and inactive virus particles.

Pharmaceutically acceptable salts can be used, for example mineral acid salts, such as hydrochlorides, hydrobromides, phosphates and sulphates, or salts of organic acids, such as acetates, propionates, malonates and benzoates.

- Pharmaceutically acceptable carriers in therapeutic compositions may additionally
30 contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents or pH buffering substances, may be present in such compositions. Such carriers enable the pharmaceutical compositions to be

formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient.

Preferred forms for administration include forms suitable for parenteral administration, e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the product is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents, such as suspending, preservative, stabilising and/or dispersing agents. Alternatively, the antibody molecule may be in dry form, for reconstitution before use with an appropriate sterile liquid.

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals. However, it is preferred that the compositions are adapted for administration to human subjects.

The pharmaceutical compositions of this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, transcutaneous (for example, see WO98/20734), subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, intravaginal or rectal routes. Hyposprays may also be used to administer the pharmaceutical compositions of the invention. Typically, the therapeutic compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared.

Direct delivery of the compositions will generally be accomplished by injection, subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Dosage treatment may be a single dose schedule or a multiple dose schedule.

It will be appreciated that the active ingredient in the composition will be an antibody molecule. As such, it will be susceptible to degradation in the gastrointestinal tract. Thus, if the composition is to be administered by a route using the gastrointestinal tract, the composition will need to contain agents which protect the antibody from degradation but which release the antibody once it has been absorbed from the gastrointestinal tract.

A thorough discussion of pharmaceutically acceptable carriers is available in Remington's Pharmaceutical Sciences (Mack Publishing Company, N.J. 1991).

It is also envisaged that the antibody of the present invention will be administered by use of gene therapy. In order to achieve this, DNA sequences encoding the heavy and light chains of the antibody molecule under the control of appropriate DNA components are introduced into a patient such that the antibody chains are expressed from the DNA sequences and assembled *in situ*.

The present invention also provides the antibody molecule of the present invention for use in treating a disease mediated by TNF α .

The present invention further provides the use of the antibody molecule according to the present invention in the manufacture of a medicament for the treatment of a disease mediated by TNF α .

The antibody molecule of the present invention may be utilised in any therapy where it is desired to reduce the level of biologically active TNF α present in the human or animal body. The TNF α may be circulating in the body or present in an undesirably high level localised at a particular site in the body.

For example, elevated levels of TNF α are implicated in acute and chronic immune and immunoregulatory disorders, infections including septic, endotoxic and cardiovascular shock, inflammatory disorders, neurodegenerative diseases, malignant diseases and alcohol induced hepatitis. Details of the numerous disorders associated with elevated levels of TNF α are set out in US-A-5 919 452. The antibody molecule of the present invention may be utilised in the therapy of diseases mediated by TNF α . Particularly relevant diseases which may be treated by the antibody molecule of the present invention include sepsis, congestive heart failure, septic or endotoxic shock, cachexia, adult respiratory distress syndrome, AIDS, allergies, psoriasis, TB, inflammatory bone disorders, blood coagulation disorders, burns, rejection episodes following organ or tissue transplant, Crohn's disease and autoimmune diseases, such as thyroiditis and rheumatoid- and osteo-arthritis.

Additionally, the antibody molecule or composition may be used: to reduce side effects associated with TNF α generation during neoplastic therapy; to eliminate or reduce shock-related symptoms associated with the treatment or prevention of graft rejection by use of an anti-lymphocyte antibody; or for treating multi-organ failure.

The antibody molecule of the present invention is preferably used for treatment of rheumatoid- or osteo-arthritis.

The present invention also provides a method of treating human or animal subjects suffering from or at risk of a disorder mediated by TNF α , the method comprising

administering to the subject an effective amount of the antibody molecule of the present invention.

The antibody molecule of the present invention may also be used in diagnosis, for example in the *in vivo* diagnosis and imaging of disease states involving elevated levels of
5 TNF α .

The present invention also provides an antibody molecule comprising a hybrid CDR comprising a truncated donor CDR sequence wherein the missing portion of the truncated donor CDR is replaced by a different sequence and forms a functional CDR. The term "hybrid CDR" as used herein means a CDR comprising a donor CDR which has been
10 truncated at one or more positions, for example at one or both of its ends. The missing portion of the truncated donor CDR is replaced by a different sequence to form a complete and functional CDR. The hybrid CDR has at least one amino acid change compared to the complete donor CDR. The sequence replacing the truncated portion of the CDR can be any sequence. Preferably the non-donor part of the CDR sequence is from the antibody from
15 which the framework regions of the antibody molecule are derived, such as a germline antibody sequence.

It has been found that antibody molecules comprising a hybrid CDR retain substantially the same binding affinity as an antibody molecule comprising complete donor CDRs. The term "substantially the same binding affinity" as used herein means at least
20 70%, more preferably at least 85% and most preferably at least 95% of the binding affinity of the corresponding antibody molecule comprising complete donor CDRs. As noted above, in certain cases, the affinity of the antibody of the invention may be greater than that of the donor antibody. The use of a hybrid CDR provides the advantages of reducing the amount of foreign (i.e. donor) sequence present in the antibody molecule and may increase
25 the binding affinity of the antibody molecule compared to the corresponding antibody molecule comprising complete donor CDRs.

Any of the CDRs of the antibody molecule can be hybrid. Preferably CDR2 of the heavy chain is hybrid in the antibody molecule.

Preferably the truncation of the donor CDR is from 1 to 8 amino acids, more
30 preferably from 4 to 6 amino acids. It is further preferred that the truncation is made at the C-terminus of the CDR.

Depending on the sequence of the truncated portion of the CDR and the sequence of the different sequence replacing the missing portion, a number of amino acid changes may

be made. Preferably at least 2 amino acid changes are made, more preferably at least 3 amino acid changes are made and most preferably at least 4 amino acid changes are made.

A particular embodiment of this aspect of the invention is an antibody according to the first aspect of the invention wherein the second CDR in the heavy chain has the
5 sequence given as SEQ ID NO:2. This has better affinity for its antigen than does the donor antibody from which part of the CDR is derived.

The present invention also provides a nucleic acid sequence which encodes the antibody molecule comprising a hybrid CDR of the present invention.

The present invention also provides an expression vector containing the nucleic acid
10 sequence encoding the antibody molecule comprising a hybrid CDR of the present invention.

The present invention also provides a host cell transformed with the vector of the present invention.

The present invention also provides a process for the production of an antibody
15 molecule comprising a hybrid CDR comprising culturing the host cell of the present invention and isolating the antibody molecule.

The present invention is further described by way of illustration only in the following examples which refer to the accompanying Figures, in which:

Figure 1 shows the framework regions of the human light chain subgroup 1
20 compared to the framework regions of the hTNF40 light chain (SEQ ID NOS:83 to 90);

Figure 2 shows the framework regions of the human heavy chain subgroup 1 and subgroup 3 compared to the framework regions of the hTNF40 heavy chain (SEQ ID NOS:91 to 98 and 106 to 109);

Figure 3 shows the amino acid sequence of the CDRs of hTNF40 (SEQ ID NOS:1
25 to 7), wherein CDR H2' is a hybrid CDR wherein the C-terminal six amino acids are from the H2 CDR sequence of a human subgroup 3 germline antibody and the amino acid changes to the sequence resulting from this hybridisation are underlined;

Figure 4 shows vector pMR15.1;

Figure 5 shows vector pMR14;

30 Figure 6 shows the nucleotide and predicted amino acid sequence of the murine hTNF40Vl (SEQ ID NO: 99);

Figure 7 shows the nucleotide and predicted amino acid sequence of the murine hTNF40Vh (SEQ ID NO:100);

Figure 8 shows the nucleotide and predicted amino acid sequence of hTNF40-gL1 (SEQ ID NO:8);

Figure 9 shows the nucleotide and predicted amino acid sequence of hTNF40-gL2 (SEQ ID NO:9);

5 Figure 10 shows the nucleotide and predicted amino acid sequence of gh1hTNF40.4 (SEQ ID NO:10);

Figure 11 shows the nucleotide and predicted amino acid sequence of gh3hTNF40.4 (SEQ ID NO:11);

Figure 12 shows vector CTIL5-gL6;

10 Figure 13 shows the structure of a compound called CDP870 comprising a modified Fab fragment derived from antibody hTNF40 covalently linked via a cysteine residue to a lysyl-maleimide linker wherein each amino group on the lysyl residue has covalently attached to it a methoxy PEG residue wherein n is about 420;

Figure 14 shows vector pTTQ9;

15 Figure 15 shows the sequence of the OmpA oligonucleotide adapter (SEQ ID NO:101);

Figure 16 shows vector pACYC184;

Figure 17 shows vector pTTO-1;

Figure 18 shows vector pTTO-2;

20 Figure 19 shows vector pDNAbEng-G1;

Figure 20 shows the oligonucleotide cassettes encoding different intergenic sequences for *E. coli* modified Fab expression (SEQ ID NOS:102 to 105);

Figure 21 shows periplasmic modified Fab accumulation of IGS variants;

Figure 22 shows vector pTTO(CDP870);

25 Figure 23 shows the disease activity score (DAS) in patients treated with different doses of CDP870 and placebo. Median and IQ ranges are presented for the per-protocol population with last observation carried forward. Small squares indicate placebo, diamonds indicate 1 mg/kg, triangles indicate 5 mg/kg and large squares indicate 20 mg/kg;

30 Figure 24 shows the tender joint count, swollen joint count, pain score, assessor's global assessment of disease activity, modified health assessment questionnaire (HAQ), C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in patients treated with different doses of CDP870 and placebo. Median and IQ range are presented for the per-

protocol population with last observation carried forward. Small squares indicate placebo, diamonds indicate 1 mg/kg, triangles indicate 5 mg/kg and large squares indicate 20 mg/kg.

EXAMPLES

5

Gene Cloning and Expression of a Chimeric hTNF40 Antibody Molecule

RNA Preparation from hTNF40 Hybridoma Cells

- Total RNA was prepared from 3×10^7 hTNF40 hybridoma cells as described below.
- 10 Cells were washed in physiological saline and dissolved in RNazol (0.2 ml per 10^6 cells). Chloroform (0.2 ml per 2 ml homogenate) was added, the mixture shaken vigorously for 15 seconds and then left on ice for 15 minutes. The resulting aqueous and organic phases were separated by centrifugation for 15 minutes in an Eppendorf centrifuge and RNA was precipitated from the aqueous phase by the addition of an equal volume of isopropanol.
- 15 After 15 minutes on ice, the RNA was pelleted by centrifugation, washed with 70% ethanol, dried and dissolved in sterile, RNase free water. The yield of RNA was 400 µg.

PCR Cloning of hTNF40 Vh and VI

- cDNA sequences coding for the variable domains of hTNF40 heavy and light chains
- 20 were synthesised using reverse transcriptase to produce single stranded cDNA copies of the mRNA present in the total RNA, followed by Polymerase Chain Reaction (PCR) on the cDNAs with specific oligonucleotide primers.

a) cDNA Synthesis

- cDNA was synthesised in a 20 µl reaction volume containing the following
- 25 reagents: 50mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM each deoxyribonucleoside triphosphate, 20 units RNasin, 75 ng random hexanucleotide primer, 2 µg hTNF40 RNA and 200 units Moloney Murine Leukemia Virus reverse transcriptase. After incubation at 42°C for 60 minutes, the reaction was terminated by heating at 95°C for 5 minutes.

30 h) PCR

Aliquots of the cDNA were subjected to PCR using combinations of primers specific for the heavy and light chains. The nucleotide sequences of the 5' primers for the heavy and light chains are shown in Tables 1 and 2 respectively. These sequences all contain, in order, a

restriction site starting 7 nucleotides from their 5' ends, the sequence GCCGCCACC (SEQ ID NO:12), to allow optimal translation of the resulting mRNAs, an initiation codon and 20-30 nucleotides based on the leader peptide sequences of known mouse antibodies (Kabat *et al.*, Sequences of proteins of immunological interest, 5th Edition, 1991, U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health).

The 3' primers are shown in Table 3. The light chain primer spans the J-C junction of the antibody and contains a restriction site for the enzyme Sp1I to facilitate cloning of the V1 PCR fragment. The heavy chain 3' primers are a mixture designed to span the J-C junction of the antibody. The 3' primer includes an ApaI restriction site to facilitate cloning. The 3' region of the primers contains a mixed sequence based on those found in known mouse antibodies (Kabat *et al.*, 1991, *supra*).

The combinations of primers described above enable the PCR products for Vh and V1 to be cloned directly into an appropriate expression vector (see below) to produce chimeric (mouse-human) heavy and light chains and for these genes to be expressed in mammalian cells to produce chimeric antibodies of the desired isotype.

Incubations (100 µl) for the PCR were set up as follows. Each reaction contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 10 pmoles 5' primer mix (Table 4), 10 pmoles 3' primer (CL12 (light chain) or R2155 (heavy chain) (Table 3)), 1 µl cDNA and 1 unit Taq polymerase. Reactions were incubated at 95°C for 5 minutes and then cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, aliquots of each reaction were analysed by electrophoresis on an agarose gel. Light chain reactions containing 5' primer mixes from light chain pools 1, 2 and 7 produced bands with sizes consistent with full length V1 fragments while the reaction from heavy chain reaction pool 3 produced a fragment with a size expected of a Vh gene. The band produced by the light chain pool 1 primers was not followed up as previous results had shown that this band corresponds to a light chain pseudogene produced by the hybridoma cell. The band produced by the light chain pool 7 primers was weaker than the band from the pool 2 primers and therefore was not followed up. Only the band from light chain reaction pool 2, which was the strongest band, was followed up.

c) Molecular Cloning of the PCR Fragments

The DNA fragments produced in the light chain reaction pool 2 were digested with the enzymes BstBI and Sp1I, concentrated by ethanol precipitation, electrophoresed on a

1.4% agarose gel and DNA bands in the range of 400 base pairs recovered. These were cloned by ligation into the vector pMR15.1 (Figure 4) that had been restricted with BstBI and SphI. After ligation, mixtures were transformed into *E. coli* LM 1035 and plasmids from the resulting bacterial colonies screened for inserts by digestion with BstBI and SphI.

- 5 Representatives with inserts from each ligation were analysed further by nucleotide sequencing.

In a similar manner, the DNA fragments produced in heavy chain reaction pool 3 were digested with HindIII and ApaI and cloned into the vector pMR14 (Figure 5) that had been restricted with HindIII and ApaI. Again, representative plasmids containing inserts
10 were analysed by nucleotide sequencing.

d) Nucleotide Sequence Analysis

Plasmid DNA from a number of isolates containing Vh inserts was sequenced using the primers R1053 (see Table 5) (which primes in the 3' region of the HCMV promoter in pMR14) and R720 (see Table 5) (which primes in the 5' region of human C – gamma 4 and
15 allows sequencing through the DNA insert on pMR14). It was found that the nucleotide sequences of the Vh insert in a number of clones were identical, except for differences in the signal peptide and J regions. This indicated that the clones examined are independent isolates arising from the use of different primers from the mixture of oligonucleotides during the PCR stage. The determined nucleotide sequence and predicted amino acid
20 sequence of the variable domain of the heavy chain of antibody hTNF40 (hTNF40Vh) are given in Figure 7 (SEQ ID NO:100).

To analyse the light chain clones, the sequence derived from priming with R1053 (see Table 5) and R684 (SEQ ID NO:62) (which primes in the 5' region of human C-kappa and allows sequencing through the DNA insert on pMR15.1) was examined. The
25 nucleotide sequence and predicted amino acid sequence of the VI genes arising from reactions in pool 2 were similarly analysed. Again it was found that the nucleotide sequences of the VI insert in a number of clones were identical, except for differences in the signal peptide and J regions, indicating that the clones examined were independent isolates arising from the use of different primers from the mixture of oligonucleotides used during
30 the PCR stage. The determined nucleotide sequence and predicted amino acid sequence of the variable domain of the light chain of antibody hTNF40 (hTNF40V1) are given in Figure 6 (SEQ ID NO:99).

TABLE 1

Oligonucleotide primers for the 5' region of mouse heavy chains.

- 5 CH1 : 5'ATGAAATGCAGCTGGGTCAT(G,C)TTCTT3' (SEQ ID NO:13)
 CH2 : 5'ATGGGATGGAGCT(A,G)TATCAT(C,G)(C,T)TCTT3' (SEQ ID NO:14)
 CH3 : 5'ATGAAG(A,T)TGTGGTTAAACTGGGTTTT3' (SEQ ID NO:15)
 CH4 : 5'ATG(G,A)ACTTTGGG(T,C)TCAGCTTG(G,A)T3' (SEQ ID NO:16)
 CH5 : 5'ATGGACTCCAGGCTCAATTTAGTTTT3' (SEQ ID NO:17)
 10 CH6 : 5'ATGGCTGTC(C,T)T(G,A)G(G,C)GCT(G,A)CTCTTCTG3' (SEQ ID NO:18)
 CH7 : 5'ATGG(G,A)ATGGAGC(G,T)GG(G,A)TCTTT(A,C)TCTT3' (SEQ ID NO:19)
 CH8 : 5'ATGAGAGTGCTGATTCTTTTGTG3' (SEQ ID NO:20)
 CH9 : 5'ATGG(C,A)TTGGGTGTGGA(A,C)CTTGCTATT3' (SEQ ID NO:21)
 CH10 : 5'ATGGGCAGACTTACATTCTCATTCCT3' (SEQ ID NO:22)
 15 CH11 : 5'ATGGATTTTGGGCTGATTTTTTTTATTG3' (SEQ ID NO:23)
 CH12 : 5'ATGATGGTGTTAAGTCTTCTGTACCT3' (SEQ ID NO:24)

Each of the above primers has the sequence 5'GCGCGCAAGCTTGCCGCCACC3' (SEQ ID NO:25) added to its 5' end.

20

TABLE 2

Oligonucleotide primers for the 5' region of mouse light chains.

- CL1 : 5'ATGAAGTTGCCTGTTAGGCTGTTGGTGCT3' (SEQ ID NO:26)
 25 CL2 : 5'ATGGAG(T,A)CAGACACACTCCTG(T,C)TATGGGT3' (SEQ ID NO:27)
 CL3 : 5'ATGAGTGTGCTCACTCAGGTCCT3' (SEQ ID NO:28)
 CL4 : 5'ATGAGG(G,A)CCCCTGCTCAG(A,T)TT(C,T)TTGG3' (SEQ ID NO:29)
 CL5 : 5'ATGGATTT(T,A)CAGGTGCAGATT(T,A)TCAGCTT3' (SEQ ID NO:30)
 CL5A : 5'ATGGATTT(T,A)CA(A,G)GTGCAGATT(T,A)TCAGCTT3' (SEQ ID NO:31)
 30 CL6 : 5'ATGAGGT(T,G)C(T,C)(T,C)TG(T,C)T(G,C)AG(T,C)T(T,C)CTG(A,G)G3' (SEQ ID NO:32)
 CL7 : 5'ATGGGC(T,A)TCAAGATGGAGTCACA3' (SEQ ID NO:33)

- CL8 : 5'ATGTGGGGA(T,C)CT(G,T)TTT(T,C)C(A,C)(A,C)TTTTTCAAT3' (SEQ ID NO:34)
- CL9 : 5'ATGGT(G,A)TCC(T,A)CA(G,C)CTCAGTTCCTT3' (SEQ ID NO:35)
- CL10 : 5'ATGTATATATGTTTGTGTCTATTTTC3' (SEQ ID NO:36)
- 5 CL11 : 5'ATGGAAGCCCCAGCTCAGCTTCTCTT3' (SEQ ID NO:37)
- CL12A : 5'ATG(A,G)AGT(T,C)(A,T)CAGACCCAGGTCTT(T,C)(A,G)T3' (SEQ ID NO:38)
- CL12B : 5'ATGGAGACACATTCTCAGGTCTTTGT3' (SEQ ID NO:39)
- CL13 : 5'ATGGATTCACAGGCCAGGTTCTTAT3' (SEQ ID NO:40)
- 10 CL14 : 5'ATGATGAGTCCTGCCAGTTCCTGTT3' (SEQ ID NO:41)
- CL15 : 5'ATGAATTTGCCTGTTTCATCTCTTGGTGCT3' (SEQ ID NO:42)
- CL16 : 5'ATGGATTTTCAATTGGTCCTCATCTCCTT3' (SEQ ID NO:43)
- CL17A : 5'ATGAGGTGCCTA(A,G)CT(C,G)AGTTCCTG(A,G)G3' (SEQ ID NO:44)
- CL17B : 5'ATGAAGTACTCTGCTCAGTTTCTAGG3' (SEQ ID NO:45)
- 15 CL17C : 5'ATGAGGCATTCTCTTCAATTCTTGGG3' (SEQ ID NO:46)

Each of the above primers has the sequence 5'GGACTGTTCGAAGCCGCCACC3' (SEQ ID NO:47) added to its 5' end.

20 TABLE 3

Oligonucleotide primers for the 3' ends of mouse Vh and Vl genes.

Light chain (CL12) :

5'GGATACAGTTGGTGCAGCATCCGTACGTTT3' (SEQ ID NO:48)

25

Heavy chain (R2155) :

5'GCAGATGGGCCCTTCGTTGAGGCTG(A,C)(A,G)GAGAC(G,T,A)GTGA3'
(SEQ ID NO:49)

TABLE 4**a) 5' Primer mixtures for light chain PCR reactions**

- pool 1 : CL2.
 5 pool 2 : CL7.
 pool 3 : CL13.
 pool 4 : CL6.
 pool 5 : CL5A, CL9, CL17A.
 pool 6 : CL8.
 10 pool 7 : CL12A.
 pool 8 : CL1, CL3, CL4, CL5, CL10, CL11, CL2B, CL14, CL15, CL16, CL17B, CL17C

b) 5' Primer mixtures for heavy chain PCR reactions

- 15 pool 1 : CH1, CH2, CH3, CH4.
 pool 2 : CH5, CH6, CH7, CH8.
 pool 3 : CH9, CH10, CH11, CH12.

Table 5**20 Primers used in nucleotide sequence analysis**

- R1053 : 5'GCTGACAGACTAACAGACTGTTCC3' (SEQ ID NO:50)
 R720 : 5'GCTCTCGGAGGTGCTCCT3' (SEQ ID NO:51)

25 Evaluation of Activities of Chimeric Genes

The activities of the chimeric genes were evaluated by expressing them in mammalian cells and purifying and quantitating the newly synthesised antibodies. The methodology for this is described below, followed by a description of the biochemical and cell based assays used for the biological characterisation of the antibodies.

30 a) Production of Chimeric hTNF40 Antibody Molecule

Chimeric antibody for biological evaluation was produced by transient expression of the appropriate heavy and light chain pairs after co-transfection into Chinese Hamster Ovary (CHO) cells using calcium phosphate precipitation.

On the day prior to transfection, semi-confluent flasks of CHO-L761 cells were trypsinised, the cells counted and T75 flasks set up each with 10^7 cells.

On the next day, the culture medium was changed 3 hours before transfection. For transfection, the calcium phosphate precipitate was prepared by mixing 1.25 ml of 0.25 M
5 CaCl_2 containing 50 μg of each of heavy and light chain expression vectors with 1.25 ml of
2 x HBS (16.36 g NaCl, 11.0 g HEPES and 0.4 g Na_2HPO_4 in 1 litre water with the pH
adjusted to 7.1 with NaOH) and adding immediately into the medium of the cells. After 3
hours at 37°C in a CO_2 incubator, the medium and precipitate were removed and the cells
shocked by the addition of 15 ml 15% glycerol in phosphate buffered saline (PBS) for 1
10 minute. The glycerol was removed, the cells washed once with PBS and incubated for 48-
96 hours in 25 ml medium containing 10 mM sodium butyrate. Antibody could be purified
from the culture medium by binding to and elution from protein A-Sepharose.

b) ELISA

For the ELISA, Nunc ELISA plates were coated overnight at 4°C with a F(ab)_2
15 fragment of a polyclonal goat anti-human Fc fragment specific antibody (Jackson
ImmunoResearch, code 109-006-098) at 5 $\mu\text{g}/\text{ml}$ in coating buffer (15 mM sodium
carbonate, 35 mM sodium hydrogen carbonate, pH 6.9). Uncoated antibody was removed
by washing 5 times with distilled water. Samples and purified standards to be quantitated
were diluted to approximately 1 $\mu\text{g}/\text{ml}$ in conjugate buffer (0.1 M Tris-HCl, pH 7.0, 0.1 M
20 NaCl, 0.2% v/v Tween 20, 0.2% w/v Hammersten casein). The samples were titrated in
the microtitre wells in 2-fold dilutions to give a final volume of 0.1 ml in each well and the
plates incubated at room temperature for 1 hour with shaking. After the first incubation
step the plates were washed 10 times with distilled water and then incubated for 1 hour as
before with 0.1 ml of a mouse monoclonal anti-human kappa (clone GD12) peroxidase
25 conjugated antibody (The Binding Site, code MP135) at a dilution of 1 in 700 in conjugate
buffer. The plate was washed again and substrate solution (0.1 ml) added to each well.
Substrate solution contained 150 μl N,N,N,N-tetramethylbenzidine (10 mg/ml in DMSO),
150 μl hydrogen peroxide (30% solution) in 10 ml 0.1 M sodium acetate/sodium citrate, pH
6.0. The plate was developed for 5-10 minutes until the absorbance at 630 nm was
30 approximately 1.0 for the top standard. Absorbance at 630 nm was measured using a plate
reader and the concentration of the sample determined by comparing the titration curves
with those of the standard.

c) **Determination of Affinity constants by BiaCore analysis.**

The binding interaction between hTNF40 and human TNF was investigated using BIA technology. An affinity purified goat polyclonal antibody, directed against the constant region of hTNF40, was immobilised on the dextran polymer sensor chip surface using
5 standard NHS/EDC chemistry. Relatively low levels (200-500 RU) of hTNF40 were captured to ensure mass transport effects were minimised. Human TNF at different concentrations was passed over the captured hTNF40 to allow assessment of the association kinetics. Following the injection of ligand, buffer was passed over the surface so that the dissociation could be measured. The association and dissociation rate constants for the
10 interaction between solid phase hTNF40 and human TNF were calculated, and a K_D value was derived.

EXAMPLE 1

CDR-Grafting of hTNF40

15 The molecular cloning of genes for the variable regions of the heavy and light chains of the hTNF40 antibody and their use to produce chimeric (mouse-human) hTNF40 antibodies has been described above. The nucleotide and amino acid sequences of the murine hTNF40 VI and Vh are shown in Figures 6 and 7 (SEQ ID NOS:99 and 100), respectively. This example describes the CDR-grafting of the hTNF40 antibody.

20

CDR-Grafting of hTNF40 Light Chain

Alignment of the framework regions of hTNF40 light chain with those of the four human light chain subgroups (Kabat *et al.*, 1991, *supra*) revealed that hTNF40 was most homologous to antibodies in human light chain subgroup 1. Consequently, for constructing
25 the CDR-grafted light chain, the framework regions chosen corresponded to those of the human group 1 consensus sequence.

A comparison of the amino acid sequences of the framework regions of murine hTNF40 and the consensus human group 1 light chains is given in Figure 1 and shows that there are 22 differences (underlined) between the two sequences. Analysis of the
30 contribution that any of these framework differences might have on antigen binding identified 2 residues for investigation; these are at positions 46 and 60. Based on this analysis, two versions of the CDR-grafted light chain were constructed. In the first of these, hTNF40-gL1 (SEQ ID NO:8), residues 46 and 60 are derived from the hTNF40 light

chain while in the second, hTNF40-gL2 (SEQ ID NO:9), all residues are human consensus except residue number 60 which is from the hTNF40 light chain.

Construction of CDR-Grafted Light Chain hTNF40-gL1.

5 The construction of hTNF40-gL1 is given below in detail. The following overlapping oligonucleotides (P7982-P7986) were used in the Polymerase Chain Reactions (PCR) to assemble a truncated grafted light chain. The assembled fragment lacks the antibody leader sequence and the first 17 amino acids of framework 1.

10 oligo 1 P7982:

5' GAATTCAGGGTCACCATCACTTGTAAGCCAGTCAGAACGTAGGTACTAAC
GTAGCCTGGTATCAGCAA3' (SEQ ID NO:52)

oligo 2 P7983:

15 5' ATAGAGGAAAGAGGCACTGTAGATGAGGGCTTTTGGGGCTTTACCTGGTTT
TTGCTGATACCAGGCTACGT3' (SEQ ID NO:53)

oligo 3 P7984:

20 5' TACAGTGCCTCTTTCCTCTATAGTGGTGTACCATACAGGTTACGCGGATCCG
GTAGTGGTACTGATTTTAC3' (SEQ ID NO:54)

oligo 4 P7985

25 5' GACAGTAATAAGTGGCGAAATCTTCTGGCTGGAGGCTACTGATCGTGAGGGT
GAAATCAGTACCACTACCG3' (SEQ ID NO:55)

oligo 5 P7986:

5' ATTTGCGCACTTATTACTGTCAACAGTATAACATCTACCCACTCACATTCGGT
CAGGGTACTAAAGTAGAAATCAAACGTACGGAATTC3' (SEQ ID NO:56)

30 Fwd P7981:

5' GAATTCAGGGTCACCATCACTTGTAAGCC3' (SEQ ID NO:57)

Bwd P7980

5'GAATTCCGTACGTTTGATTTCTACTTTAGT3' (SEQ ID NO:58),

- A PCR reaction, 100 µl, was set up containing, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 2 pmoles of P7982, P7983, P7984, P7985, P7986, 10 pmoles of P7980, P7981 and 1 unit of Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, each reaction was analysed by electrophoresis on an agarose gel and the PCR fragment excised from the gel and recovered using a Mermaid Kit.
- 10 The recovered fragment was restricted with the enzymes BstEII and SphI in the appropriate buffer. The resulting product was finally electrophoresed on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into vector CTIL5-gL6 (Figure 12), that had previously been digested with the same enzymes. The above vector provides the missing antibody leader sequence and the first 17 amino acids of framework 1.
- 15 The ligation mixture was used to transform E. coli strain LM1035 and resulting colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the V1 region of hTNF40-gL1 is shown in Figure 8 (SEQ ID NO:8).

20 **Construction of CDR-Grafted Light Chain hTNF40-gL2.**

hTNF40-gL2 (SEQ ID NO:9) was constructed using PCR. The following oligonucleotides were used to introduce the amino acid changes:

- R1053: 5'GCTGACAGACTAACAGACTGTTCC3' (SEQ ID NO:59)
- 25 R5350: 5'TCTAGATGGCACACCATCTGCTAAGTTTGATGCAGCATAGAT
CAGGAGCTTAGGAGC3' (SEQ ID NO:60)
- R5349: 5'GCAGATGGTGTGCCATCTAGATTCAAGTGGCAGTGGATCA
30 GGCACAGACTTTACCCTAAC3' (SEQ ID NO:61)
- R684: 5'TTCAACTGCTCATCAGAT3' (SEQ ID NO:62)

Two reactions, each 20 μ l, were set up each containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 0.1 μ g hTNF40-gL1, 6 pmoles of R1053/R5350 or R5349/R684 and 0.25 units Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, each reaction was analysed by electrophoresis on an agarose gel and the PCR fragments excised from the gel and recovered using a Mermaid Kit.

Aliquots of these were then subjected to a second round of PCR. The reaction, 100 μ l, contained 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% w/v gelatin, 1/5 of each of the PCR fragments from the first set of reactions, 30 pmoles of R1053 and R684 and 2.5 units Taq polymerase. Reaction temperatures were as above. After the PCR, the mixture was extracted with phenol/chloroform and then with chloroform and precipitated with ethanol. The ethanol precipitate was recovered by centrifugation, dissolved in the appropriate buffer and restricted with the enzymes BstEII and SphI. The resulting product was finally electrophoresed on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into the vector pMR15.1 (Figure 4) that had previously been digested with the same enzymes.

The ligation mixture was used to transform *E. coli* LM1035 and resulting colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the VI region of hTNF40-gL2 is shown in Figure 9 (SEQ ID NO:9).

CDR-Grafting of hTNF40 Heavy Chain

CDR-grafting of hTNF40 heavy chain was accomplished using the same strategy as described for the light chain. hTNF40 heavy chain was found to be most homologous to human heavy chains belonging to subgroup 1 and therefore the consensus sequence of the human subgroup 1 frameworks was chosen to accept the hTNF40 heavy chain CDRs.

To investigate the requirement of a homologous human framework to act as an acceptor framework for CDR grafting, a second framework, human group 3, was selected to humanise hTNF40 heavy chain.

A comparison of hTNF40 with the two different frameworks region is shown in Figure 2 where it can be seen that hTNF40 differs from the human subgroup 1 consensus at 32 positions (underlined) and differs from the human subgroup 3 consensus at 40 positions

(underlined). After analysis of the contribution that any of these might make to antigen binding, residues 28, 38, 46, 67, 69 and 71 were retained as donor in the CDR-grafted heavy chain gh1hTNF40.1, using the group 1 framework. Residues 27, 28, 30, 48, 49, 69, 71, 73, 76 and 78 were retained as donor in the CDR-grafted heavy chain, gh3hTNF40.4 using the group 3 framework. Residues 28, 69 and 71 were retained as donor in the CDR-grafted heavy chain, gh1hTNF40.4 using the group 1 framework.

Construction of CDR-Grafted Heavy Chain gh1hTNF40.4

gh1hTNF40.4 (SEQ ID NO:10) was assembled by subjecting overlapping oligonucleotides to PCR in the presence of the appropriate primers. The following oligonucleotides were used in the PCR:

Group 1 graft

oligo 1 P7989:

5'GAAGCACCAGGCTTCTTAACCTCTGCTCCTGACTGGACCAGCTGCACCTGAG
AGTGCACGAATTC3' (SEQ ID NO:63)

oligo 2 P7990:

5'GGTTAAGAAGCCTGGTGCTTCCGTCAAAGTTTCGTGTAAGGCCTCAGGCTAC
GTGTTACAGACTATGGTA3' (SEQ ID NO:64)

oligo 3 P7991:

5'CCAACCCATCCATTTACAGGCCTTGTCCTGGGGCCTGCTTGACCCAATTCATAC
CATAGTCTGTGAACACGT3' (SEQ ID NO:65)

oligo 4 P7995:

5'GGCCTGAAATGGATGGGTGGATTAATACTTACATTGGAGAGCCTATTTATGT
TGACGACTTCAAGGGCAGATTCACGTTC3' (SEQ ID NO:66)

oligo 5 P7992:

5'CCATGTATGCAGTGCCTTGTGGAGGTGTCTAGAGTGAACGTGAATCTGCCCTT
GAA3' (SEQ ID NO:67)

oligo 6 P7993:

5'CCACAAGCACTGCATACATGGAGCTGTCATCTCTGAGATCCGAGGACACCGC
AGTGTACTAT3' (SEQ ID NO:68)

5

oligo 7 P7994:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCATGGCATAAGATCTGTATCCTCTAG
CACAATAGTACACTGCGGTGTCCTC3' (SEQ ID NO:69)

10 Fwd: P7988:

5'GAATTCGTGCACTCTCAGGTGCAGCTGGTC3' (SEQ ID NO:70)

Bwd P7987:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCAT3' (SEQ ID NO:71)

15

The assembly reaction, 100 μ l, contained 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 2 pmole of each of p7989, p7990, p7991, p7995, p7992, p7993 and p7994, 10 pmoles of each of p7988 and p7987 and 1 unit Taq polymerase. Reactions were cycled through 94°C for 1
20 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, the reaction was extracted with phenol/chloroform (1/1), then with chloroform and precipitated with ethanol. After centrifugation, the DNA was dissolved in the appropriate restriction buffer and digested with ApaLI and KpnI. The resulting fragment was isolated from an agarose gel and ligated into pMR14 (Figure 5) that had previously been digested with the same
25 enzymes. pMR14 contains the human gamma 4 heavy chain constant region when pMR14 is cleaved with ApaLI and KpnI, the cleaved vector is able to receive the digested DNA such that the 3' end of the digested DNA joins in reading frame to the 5' end of the sequence encoding the gamma 4 constant region. Therefore, the heavy chain expressed from this vector will be a gamma 4 isotype. The ligation mixture was used to transform *E.*
30 *coli* LM1035 and resulting bacterial colonies screened by restriction digest and nucleotide sequence analysis. In this way, a plasmid was identified containing the correct sequence for gh1hTNF40.4 (Figure 10) (SEQ ID NO:10).

Construction of CDR-Grafted Heavy Chain gh3hTNF40.4

gh3hTNF40.4 (SEQ ID NO:11) was assembled by subjecting overlapping oligonucleotides to PCR in the presence of the appropriate primers. The following
5 oligonucleotides were used in the PCR:

Group 3 graft**oligo 1 P7999:**

10 5'GATCCGCCAGGCTGCACGAGACCGCCTCCTGACTCGACCAGCTGAACCTCAG
AGTGCACGAATTC3' (SEQ ID NO:72)

oligo 2 P8000:

5'TCTCGTGCAGCCTGGCGGATCGCTGAGATTGTCCTGTGCTGCATCTGGTTACG
15 TCTTCACAGACTATGGAA3' (SEQ ID NO:73)

oligo 3 P8001

5'CCAACCCATCCATTTTCAGGCCCTTTCCCGGGGCCTGCTTAACCCAATTCATTC
CATAGTCTGTGAAGACGT3' (SEQ ID NO:74)

20

oligo 4 P7995:

5'GGCCTGAAATGGATGGGTTGGATTAATACTTACATTGGAGAGCCTATTTATGT
TGACGACTTCAAGGGCAGATTACGTTTC3' (SEQ ID NO:66)

25 oligo 5 P7997:

5'GGAGGTATGCTGTTGACTTGGATGTGTCTAGAGAGAACGTGAATCTGCCCTT
GAA3' (SEQ ID NO:75)

oligo 6 P7998:

30 5'CCAAGTCAACAGCATACCTCCAAATGAATAGCCTGAGAGCAGAGGACACCGC
AGTGTACTAT3' (SEQ ID NO:76)

oligo 7 P7993:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCATGGCATAAGATCTGTATCCTCTAG
CACAATAGTACACTGCGGTGTCCTC3' (SEQ ID NO:77)

5 Fwd P7996:

5'GAATTCGTGCACTCTGAGGTTTCAGCTGGTC3' (SEQ ID NO:78)

Bwd P7987:

5'GAATTCGGTACCCTGGCCCCAGTAGTCCAT3' (SEQ ID NO:71)

10

The assembly reaction, 100 µl, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 2 pmole of each of p7999, p8000, p8001, p7995, p7997, p7998 and p7993, 10 pmoles of each of p7996 and p7987 and 1 unit Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, the reaction was extracted with phenol/chloroform (1/1), then with chloroform and precipitated with ethanol. After centrifugation, the DNA was dissolved in the appropriate restriction buffer and digested with ApaLI and KpnI. The resulting fragment was isolated from an agarose gel and ligated into pMR14 (Figure 5) that had previously been digested with the same enzymes. pMR14 contained the human gamma 4 heavy chain constant region. When pMR14 is cleaved with ApaLI and KpnI, the cleaved vector is able to receive the digested DNA such that the 3' end of the digested DNA joins in reading frame to the 5' end of the sequence encoding the gamma 4 constant region. Therefore, the heavy chain expressed from this vector will be a gamma 4 isotype. The ligation mixture was used to transform *E. coli* LM1035 and resulting bacterial colonies screened by restriction digestion and nucleotide sequence analysis. In this way, a plasmid was identified containing the correct sequence for gh3hTNF40.4 (SEQ ID NO:11) (Figure 11).

20

25

Production of CDR-Grafted Modified Fab Fragment.

30

A CDR-grafted, modified Fab fragment, based on antibody hTNF40, was constructed using the *E. coli* vector pTTO-1. The variable regions of antibody hTNF40 are sub-cloned into this vector and the intergenic sequence optimised to create pTTO(CDP870). The pTTO expression vector is designed to give rise to soluble,

periplasmic accumulation of recombinant proteins in *E. coli*. The main features of this plasmid are:

- (i) tetracycline resistance marker - antibiotic not inactivated by the product of resistance gene, hence selection for plasmid-containing cells is maintained;
- 5 (ii) low copy number - origin of replication derived from plasmid p15A, which is compatible with plasmids containing *colE1* derived replicons;
- (iii) strong, inducible *tac* promoter for transcription of cloned gene(s);
- (iv) *lacI^q* gene - gives constitutive expression of the *lac* repressor protein, maintaining the *tac* promoter in the repressed state until induction with IPTG / allolactose;
- 10 (v) OmpA signal sequence - gives periplasmic secretion of cloned gene(s); and
- (vi) translational coupling of OmpA signal sequence to a short *lacZ* peptide, giving efficient initiation of translation.

The vector has been developed for expression of modified Fab fragments from a
15 dicistronic message by the design of a method to select empirically the optimum intergenic sequence from a series of four purpose-built cassettes. The application of this in the construction of pTTO(CDP870) is described.

Materials and Methods

20

DNA techniques

Standard procedures were used for protocols including DNA restriction, agarose gel electrophoresis, ligation and transformation. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs or Boehringer Mannheim, and were
25 used according to the supplier's recommendations. DNA fragments were purified from agarose using the GeneClean protocol (BIO 101). Oligonucleotides were supplied by Oswel Oligonucleotide Service and were synthesized at the 40 nm scale. Plasmid DNA was isolated using Plasmid DNA Mini / Midi kits from Qiagen. PCR was performed using Perkin Elmer 'Amplitaq' as recommended. DNA sequencing was performed using the
30 Applied Biosystems Taq cycle sequencing kit.

Shake flask induction

E. coli W3110 cultures were grown in L-broth supplemented with tetracycline (7.5 µg/ml). For inductions, fresh overnight cultures (grown at 30°C) were diluted to OD₆₀₀ of

0.1 into 200 ml L-broth in a 2 L baffled flask and were grown at 30°C in an orbital incubator. At OD₆₀₀ of 0.5, IPTG was added to 200 µM. Samples (normalised for OD) were taken at intervals.

5 Periplasmic Extraction

Culture samples were chilled on ice (5 minutes) then cells were harvested by centrifugation. Following resuspension in extraction buffer (100 mM Tris.HCl, 10 mM EDTA, pH 7.4) samples were incubated overnight at 30°C, then clarified by centrifugation.

10 Assembly Assay

Modified Fab concentrations were determined by ELISA. Plates were coated at 4°C overnight with anti-human Fd 6045 (2 µg/ml in coating buffer, physiological saline, 100 µl per well). After washing, 100 µl of sample was loaded per well; purified A5B7 gamma-1 Fab', initially at 2 µg/ml, was used as a standard. Samples were serially diluted 2-fold across the plate in sample conjugate buffer (per litre: 6.05 g trisaminomethane; 2.92 g NaCl; 0.1 ml Tween-20; 1 ml casein (0.2%)); plates were incubated for 1 hour at room temperature, with agitation. Plates were washed and dried, then 100 µl of anti-human C-kappa (GD12)-peroxidase was added (diluted in sample conjugate buffer). Incubation was carried out at room temperature for 1 hour with agitation. Plates were washed and dried, then 100 µl of substrate solution was added (10 ml sodium acetate/citrate solution (0.1 M pH 6); 100 µl H₂O₂ solution; 100 µl tetramethylbenzidine solution (10 mg/ml in dimethylsulphoxide)). Absorbance at 630 nm was read 4 - 6 minutes after substrate addition.

25 Construction of Plasmid pTTO-1

(a) Replacement of the pTTO9 Polylinker

Plasmid pTTO9 was obtained from Amersham and is shown in Figure 14. An aliquot (2 µg) was digested with restriction enzymes SalI and EcoRI, the digest was run on a 1% agarose gel and the large DNA fragment (4520 bp) was purified. Two oligonucleotides were synthesized which, when annealed together, encode the OmpA polylinker region shown in Figure 15. This sequence has cohesive ends which are compatible with the SalI and EcoRI ends generated by restriction of pTTO9. By cloning this oligonucleotide 'cassette' into the pTTO9 vector, the SalI site is not regenerated, but the

EcoRI site is maintained. The cassette encodes the first 13 amino acids of the signal sequence of the *E. coli* outer-membrane protein Omp-A, preceded by the Shine Dalgarno ribosome binding site of the OmpA gene. In addition restriction sites for enzymes XbaI, MunI, StyI and SphI are present. The MunI and StyI sites are within the coding region of the OmpA signal sequence and are intended as the 5' cloning sites for insertion of genes. The two oligonucleotides which make up this cassette were annealed together by mixing at a concentration of 5 pmoles/ μ l and heating in a waterbath to 95°C for 3 minutes, then slow cooling to room temperature. The annealed sequence was then ligated into the SalI / EcoRI cut pTTQ9. The resulting plasmid intermediate, termed pTQOmp, was verified by DNA sequencing.

(b) Fragment Preparation and Ligation

Plasmid pTTO-1 was constructed by ligating one DNA fragment from plasmid pACYC184 to two fragments generated from pTQOmp. Plasmid pACYC184 was obtained from New England Biolabs, and a restriction map is shown in Figure 16. An aliquot (2 μ g) was digested to completion with restriction enzyme StyI, then treated with Mung Bean Nuclease; this treatment creates blunt ends by cutting back 5' base overhangs. Following phenol extraction and ethanol precipitation, the DNA was restricted with enzyme PvuII, generating fragments of 2348, 1081, 412 and 403 bp. The 2348 bp fragment was purified after agarose gel electrophoresis. This fragment encodes the tetracycline resistance marker and the p15A origin of replication. The fragment was then treated with calf intestinal alkaline phosphatase to remove 5' terminal phosphates, thereby preventing the self-ligation of this molecule.

An aliquot (2 μ g) of plasmid pTQOmp was digested with enzymes SspI and EcoRI, and the 2350 bp fragment was purified from unwanted fragments of 2040 bp and 170 bp following agarose gel electrophoresis; this fragment encodes the transcriptional terminator region and the lacI^q gene. Another aliquot (2 μ g) of pTQOmp was digested with EcoRI and XmnI, generating fragments of 2289, 1670, 350 and 250 bp. The 350 bp fragment, encoding the tac promoter, OmpA signal sequence and multicloning site, was gel purified.

The three fragments were then ligated, using approximately equimolar amounts of each fragment, to generate the plasmid pTTO-1. All cloning junctions were verified by DNA sequencing. The restriction map of this plasmid is shown in Figure 17. Plasmid pTTO-2 was then created by insertion of DNA encoding the human Ig light chain kappa constant domain. This was obtained as a SphI - EcoRI restriction fragment from plasmid

pHC132, and inserted into the corresponding sites in pTTO-1. Plasmid pTTO-2 is shown in Figure 18.

Insertion of humanized hTNF40 variable regions into pTTO-2

5 The variable light chain region hTNF40gL1 (SEQ ID NO:8) was obtained by PCR 'rescue' from the corresponding vector for mammalian cell expression pMR10.1. The OmpA leader sequence replaces the native Ig leader. The sequence of the PCR primers is shown below:

10 5' primer:

CGCGCGGCAATTGCAGTGGCCTTGGCTGGTTTCGCTACCGTAGCGCAAG
CTGACATTCAAATGACCCAGAGCCC (SEQ ID NO:79)

3' primer: TTCAACTGCTCATCAGATGG (SEQ ID NO:80)

15

Following PCR under standard conditions, the product was purified, digested with enzymes MunI and SphI then gel purified. The purified fragment was then inserted into the MunI / SphI sites of pTTO-2 to create the light chain intermediate pTTO(hTNF40L).

20 The variable heavy chain region of gh3hTNF40.4 was obtained in the same way from the vector pGamma-4. The sequence of the PCR primers is shown below:

5' primer:

GCTATCGCAATTGCAGTGGCGCTAGCTGGTTTCGCCACCGTGGCGCAAG
CTGAGGTTTCAGCTGGTCGAGTCAGGAGGC (SEQ ID NO:81)

25

3' primer: GCCTGAGTTCCACGACAC (SEQ ID NO:82)

30 Following PCR the product was purified, digested with enzymes NheI and ApaI then sub-cloned into the vector pDNAEng-G1 (Figure 19). After verification by DNA sequencing, the heavy chain was restricted with enzyme EcoRI and sub-cloned into the EcoRI site of pTTO(hTNF40L) to create the *E. coli* expression plasmid pTTO(hTNF40).

Optimisation of Intergenic Sequence for Modified Fab Expression

In the pTTO vector, modified Fab expression occurs from a dicistronic message encoding first light chain then heavy chain. The DNA sequence between the two genes (intergenic sequence, IGS) can influence the level of expression of the heavy chain by affecting the rate of translational initiation. For example, a short intergenic sequence may result in translational coupling between the light and heavy chains, in that the translating ribosome may not fully dissociate from the mRNA after completing light chain synthesis before initiating heavy chain synthesis. The 'strength' of any Shine Dalgarno (SD) ribosome binding site (homology to 16S rRNA) can also have an effect, as can the distance and sequence composition between the SD and the ATG start codon. The potential secondary structure of mRNA around the ATG is another important factor; the ATG should be in a 'loop' and not constrained within a 'stem', while the reverse applies to the SD. Thus by modifying the composition and length of the IGS it is possible to modify the strength of translational initiation and therefore the level of heavy chain production. It is likely that an optimum rate of translational initiation needs to be achieved to maximise expression of the heavy chain of a given modified Fab. For example, with one modified Fab, a high level of expression may be tolerated, but for a different modified Fab with different amino acid sequence, a high level of expression might prove toxic, perhaps because of different efficiencies of secretion or folding. For this reason, a series of four intergenic sequences were designed (Figure 20), permitting the empirical determination of the optimum IGS for the hTNF40-based modified Fab. IGS1 and IGS2 have very short intergenic sequences (-1 and +1 respectively) and might be expected to give closely coupled translation; the SD sequences (underlined) are subtly different. These two sequences will most likely confer a high level of translational initiation. IGS3 and IGS4 have a longer distance between start and stop codons (+13) and differ in their sequence composition; IGS3 has a 'stronger' SD sequence. All sequences were studied for secondary structure (using m/fold program) and 'optimised' as far as possible; however, with tight coupling of translation of the two chains the lack of ribosomal dissociation means that the mRNA may not be 'naked', preventing secondary structure formation.

Cloning of IGS variants

The IGS cassettes shown in Figure 20 have flanking SacI and MunI cloning sites. They were built by annealing complementary oligonucleotide pairs. A vector fragment was prepared by digesting pTTO(hTNF40) with SacI and NotI, and a heavy chain fragment was

prepared by digesting pDNA_{EngG1(hTNF40H)} with *MunI* and *NotI*. Three-way ligations were then performed, using equimolar amounts of the two restriction fragments and approximately 0.05 pmoles of each annealed oligo cassette. This created the four expression plasmids pTTO(hTNF40 IGS-1), pTTO(hTNF40 IGS-2), pTTO(hTNF40 IGS-3), pTTO(hTNF40 IGS-4).

Shake flask expression analysis

The four plasmids were transformed into *E. coli* strain W3110, along with the original expression construct, and then analysed for expression in shake flasks as described.

10 The results of a typical experiment are shown in Figure 21. The different intergenic sequences confer different expression profiles. IGS1 and IGS2 accumulate periplasmic modified Fab rapidly with a peak at 1 hour post induction, after which the level recovered falls. The peak is greater and the fall sharper for IGS1. These results are consistent with a high level of synthesis, as expected for close translational coupling for these constructs.

15 IGS1 apparently confers a higher level of heavy chain expression than does IGS2. In this instance, it appears that this high level of expression is poorly tolerated, since periplasmic expression levels fall after the 1 hour peak. This is seen on the growth profile of the IGS1 culture (not shown), which peaks at 1 hour post induction before falling, suggesting cell death and lysis. IGS3 accumulates modified Fab more slowly but peaks at 2 hours post

20 induction with a higher peak value (325 ng/ml/OD), before levels fall. The growth of this culture continued to 3 hours post induction and reached a higher peak biomass (not shown). This is consistent with a lower level of heavy chain synthesis. IGS4 accumulates material at a slower rate still and fails to reach the high peak of productivity of the other 3 constructs. All IGS variants out-perform the original vector significantly. The hypothesis

25 that the different IGS sequences confer different rates of translational initiation is supported by these experimental results. For the hTNF40-based modified Fab it appears that a high rate of heavy chain translational initiation is poorly tolerated and is therefore not optimal. A slower rate, as conferred by IGS3, results in better growth characteristics and consequently a better yield accumulates over time.

30 Following comparison of productivity in the fermenter the IGS3 construct was selected as the highest yielding and was termed pTTO(CDP870) – see Figure 22.

The heavy chain encoded by the plasmid pTTO(CDP870) has the sequence given in SEQ ID NO:115 and the light chain has the sequence given in SEQ ID NO:113.

PEGylation of CDR-Grafted, hTNF40-based Modified Fab.

The purified modified Fab is site-specifically conjugated with a branched molecule of PEG. This is achieved by activation of a single cysteine residue in a truncated hinge region of the modified Fab, followed by reaction with (PEG)-lysyl maleimide as previously described (A.P. Chapman *et al.*, Nature Biotechnology 17, 780-783; 1999). The PEGylated molecule is shown in Figure 13 and is called compound CDP870:

Efficacy of PEGylated CDR-Grafted, hTNF40-based Modified Fab (CDP870) in Treating Rheumatoid Arthritis.

CDP870 has a long half life of approximately 11 days.

We evaluated the safety and efficacy of intravenous CDP870 in a randomised double-blind placebo-controlled dose escalating trial in patients with RA.

Methods

15 Patients:

Patients aged between 18 and 75 years old and who satisfied the 1987 revised American College of Rheumatology (ACR) diagnostic criteria for rheumatoid arthritis (RA) (Arnett *et al.*, Arthritis Rheum., 31, 315-324, 1988) were recruited from outpatient Rheumatology clinics at London, Cambridge, Norfolk and Norwich (United Kingdom). Patients were required to have clinically active disease as defined by having at least 3 of the following criteria: ≥ 6 painful or tender joints; ≥ 45 minutes of early morning stiffness; and erythrocyte sedimentation rate (ESR) ≥ 28 mm/hr. They must have failed to respond to at least one Disease Modifying Anti-Rheumatic Drug (DMARD) and have been off treatment for at least 4 weeks. Corticosteroids were permitted if the dose was ≥ 7.5 mg/day of prednisolone. Pregnant women, nursing women and women of childbearing potential not using an effective method of contraception were excluded. Patients were also excluded if they had a previous history of malignancy, concomitant severe uncontrolled medical conditions, previous failure of TNF α -neutralizing therapy or allergy to polyethylene glycol. Written informed consent was obtained from each patient before enrolment. The study was approved by the local research ethics committees.

Treatment protocol:

36 RA patients were divided into 3 groups, each to receive an increasing dose of the trial drug (1, 5 or 20mg/kg). Each group of 12 was randomly divided into 8 to receive CDP870 and 4 to receive placebo. CDP870 was given as a single intravenous infusion (100 ml in total) over 60 minutes. Placebo (sodium acetate buffer) was given similarly as a single intravenous infusion of 100 ml over 60 minutes. Treatment was given on an outpatient basis. After 8 weeks, all patients had the opportunity to receive an infusion of either 5 or 20 mg/kg of CDP870 in open fashion.

10 *Clinical assessment:*

RA disease activity was assessed based on the World Health Organization and International League of Associations for Rheumatology (Boers *et al.*, J. Rheumatol – Supplement, 41, 86-89, 1994) and European League Against Rheumatism (EULAR) (Scott *et al.*, Clin. Exp. Rheumatol., 10, 521-525, 1992) core data sets with 28 joint counts. Changes in disease activity were assessed by Disease Activity Score (Prevoo *et al.*, Arthritis Rheum., 38, 44-48, 1995) and the ACR responses criteria (Felson *et al.*, Arthritis Rheum., 38, 727-735, 1995). Assessments were carried out before treatment and at 1, 2, 4, 6 and 8 weeks after therapy. Patients were also assessed for safety and tolerance of the study drug. Haematology, biochemistry, anti-CDP870 antibodies and adverse events were assessed at each visit.

CDP870 plasma concentration and anti-CDP870 antibodies:

CDP870 was measured by enzyme-linked immunosorbent assay (ELISA). Serial dilutions of patients' plasma were incubated in microtitre plates (Nunc) coated with recombinant human TNF α (Strathmann Biotech GmbH, Hannover). Captured CDP870 was revealed with horseradish peroxidase conjugated goat anti-human kappa light chain (Cappel, ICN) followed by tetramethylbenzidine (TMB) substrate.

Antibodies to CDP870 was screened (at 1/10 plasma dilution) using a double antigen sandwich ELISA with biotinylated CDP870 as the second layer. Bound antibodies were revealed using HRP-streptavidin and TMB substrate. The assay was calibrated using a hyperimmune rabbit IgG standard. A unit of activity is equivalent to 1 μ g of the rabbit standard.

Statistical Analysis

The study was exploratory in nature and the sample size was based on previous experience with similar agents. Efficacy of CDP870 was analysed by calculating disease activity score (DAS) and ACR20/50 responses for intention to treat and per-protocol using a closed testing procedure. The disease activity score was calculated as follows: $DAS = 0.555 \times \text{square root of (28 tender joints)} + 0.284 \times \text{square root of (28 swollen joints)} + 0.7 \times \ln(\text{ESR}) + 0.0142 \times (\text{patient's global assessment})$. First, the pooled active groups were compared to placebo. If this comparison was significant at the 5% level, each dosage group was compared to placebo. All comparisons were two tailed with a significance level of 5%.

All P-values were derived from exploratory analysis and should not be used for inferential interpretation.

Results

Demography:

36 patients with RA were recruited. Their demographic details are given in Table 6. The mean age was 56 years and 30 patients were female. The mean duration of RA was 13 years and 21 patients were rheumatoid factor positive. Patients in the different groups have similar demographic characteristics. In the blinded dosing period, 6/12 placebo-treated patients withdrew from the study for deteriorating RA ≥ 4 , weeks after dosing. 2/24 CDP870-treated patients withdrew, both in the 1 mg/kg group, for deteriorating RA/lost to follow up > 4 weeks after dosing. The difference was statistically significant ($p=0.009$, Fisher exact test).

Table 6: Demographic details (mean \pm standard deviation)

	Number	Sex (M:F)	Age	Duration of Disease	Rheuma- toid Factor	Number of previous DMARDs
Placebo	12	1:11	51 \pm 9	12 \pm 8	8(67%)	5 \pm 1
1 mg/kg	8	1:7	59 \pm 7	12 \pm 7	4(50%)	4 \pm 1
5m g/kg	8	2:6	54 \pm 13	13 \pm 5	5(63%)	5 \pm 2
20 mg/kg	8	2:6	61 \pm 11	14 \pm 13	4(50%)	4 \pm 2

Clinical Efficacy:

The proportion of patients with ACR20 improvement for the per-protocol population with last observation carried forward was 16.7, 50, 87.5 and 62.5% after placebo, 1, 5 and 20 mg/kg CDP870 (combined treatment effect $p=0.012$) at 4 weeks and 16.7, 25, 75 and 75% ($p=0.032$) at 8 weeks. Reduction in DAS scores (median) for the per-protocol population with last observation carried forward was 0.15, 1.14, 1.91 and 1.95 after placebo, 1, 5 and 20 mg/kg CDP870 (combined treatment effect $p=0.001$) at 4 weeks and 0.31, 0.09, 2.09 and 1.76 ($p=0.008$) at 8 weeks (Figure 23). Changes in individual components of the World Health Organization and International League of Associations for Rheumatology core data set are shown in Figure 24.

Following the open label dose of CDP870, similar beneficial effects were achieved. Of the 36 patients recruited into the study, 32 received a second infusion of CDP870. The proportion of patients with ACR20 improvement from pre-first infusion was 72.2 and 55.6% after 5 and 20 mg/kg CDP870 at 4 weeks and 55.6 and 66.7% at 8 weeks.

Adverse Events

Treatment was well tolerated with no infusion-related reaction. No allergic reaction or skin rash was reported. In the double-blind phase, there were 19, 38, 8 and 14 adverse events in the placebo, 1, 5 and 20 mg/kg groups respectively. The commonest was headache with 9 episodes in 5 patients (1 placebo, 3 at 1 mg/kg, 1 at 20 mg/kg). One patient who received placebo and 3 patients who received CDP870 (1 at 5 mg/kg and 2 at 20 mg/kg) developed lower respiratory tract infections. These were reported as mild or moderate. They were treated with oral antibiotics and resolved over 1-2 week period. Three patients each in the 1 and 5 mg/kg groups and one in the 20 mg/kg group developed a urinary tract infection 1-2 months after CDP870 treatment. One adverse event was described as severe which was an episode of neck pain occurring 3 days after infusion with 1 mg/kg. Increase in anti-nuclear antibody was seen in 4 patients: 1 in the placebo group (negative to 1/40), 2 in the 1 mg/kg group (negative to 1/40, negative to 1/80) and 1 in the 20 mg/kg group (negative to 1/40). No change was found in anti-DNA or anti-cardiolipin antibodies.

CDP870 Plasma Concentration and Anti-CDP870 levels

As expected, for all dose levels of CDP870, the peak plasma concentration occurred at the end of infusion and was dose proportional with plasma concentration declining slowly thereafter. The plasma concentration profile of CDP870 appeared very similar to that previously observed in volunteers where the half-life was calculated to be approximately 14 days. On re-dosing, a similar profile to single dose infusion was observed.

Following a single intravenous infusion, anti-CDP870 levels were low or undetectable.

Discussion

Neutralizing TNF α is an effective treatment strategy in RA. Currently, this requires the use of biological agents, such as a chimeric mAb or a soluble receptor/human Fc fusion protein, which are expensive to manufacture. A therapeutic TNF α neutralizing agent needs to bind TNF α with high affinity and have a long plasma half-life, low antigenicity and high tolerability and safety. It also needs to be accessible to all patients with RA who would benefit from TNF α blockade. One technology that could achieve these objectives is the conjugation with polyethylene glycol of a TNF α binding antibody fragment made in *E. coli*. In this preliminary study, we find that CDP870, a PEGylated, anti-TNF α , modified Fab, is effective and well tolerated by patients with RA.

In vitro studies have shown that CDP870 has similar TNF α neutralizing activity to the murine anti-TNF α parent antibody. This study confirms that CDP870 reduced inflammation and improved symptoms in RA. Clinical improvement as measured by the ACR20 response criteria in the 5 and 20 mg/kg groups (75%, 75%) was comparable to etanercept (60%) (Moreland *et al.*, *Annals Int. Med.*, 130, 478-486, 1999) and infliximab (50%) (Maini *et al.*, *Lancet*, 354, 1932-1939, 1999). At the middle and highest dosage levels tested, the therapeutic effect lasted 8 weeks which is comparable to previous other mAbs (Elliott *et al.*, *Lancet*, 344, 1105-1110, 1994 and Rankin *et al.*, *Br. J. Rheumatol.*, 34, 334-342, 1995). Previous study has shown that the therapeutic effect of anti-TNF α antibody is related to its plasma half-life and the generation of circulating antibodies (Maini *et al.*, *Arthritis Rheum.* 38, (Supplement) : S186 1995 (Abstract)). Our study showed that CDP870 has a plasma half-life of 14 days which is equivalent to that of a whole antibody

(Rankin *et al.*, (*supra*)) and much longer than the half-life of unconjugated Fab' fragments. Further, CDP870 generated only very low levels of antibody response.

One of the important objectives of this study is to examine the tolerability and safety of administering this PEGylated Fab'. In our study, CDP870 appears well tolerated.

- 5 Although further study will be needed to assess long-term toxicity, especially the risk of demyelinating disease, infection and skin rashes that have been reported with etanercept and infliximab.

In summary, CDP870 is therapeutically effective in RA and was well tolerated in this short-term study.

- 10 It should be understood that the above-described examples are merely exemplary and do not limit the scope of the present invention as defined in the following claims.

CLAIMS

1. An antibody molecule having specificity for human TNF α , comprising a heavy
5 chain wherein the variable domain comprises a CDR having the sequence given as H1 in
Figure 3 (SEQ ID NO:1) for CDRH1, as H2' in Figure 3 (SEQ ID NO:2) or as H2' in
Figure 3 (SEQ ID NO:7) for CDRH2 or as H3 in Figure 3 (SEQ ID NO:3) for CDRH3.
2. An antibody molecule having specificity for human TNF α , comprising a light
10 chain wherein the variable domain comprises a CDR having the sequence given as L1 in
Figure 3 (SEQ ID NO:4) for CDRL1, as L2 in Figure 3 (SEQ ID NO:5) for CDRL2 or as
L3 in Figure 3 (SEQ ID NO:6) for CDRL3.
3. The antibody molecule of claim 1 or claim 2 comprising a heavy chain wherein the
15 variable domain comprises a CDR having the sequence given in SEQ ID NO:1 for CDRH1,
SEQ ID NO:2 or SEQ ID NO:7, for CDRH2 or SEQ ID NO:3 for CDRH3 and a light chain
wherein the variable domain comprises a CDR having the sequence given in SEQ ID NO:4
for CDRL1, SEQ ID NO:5 for CDRL2 or SEQ ID NO:6 for CDRL3.
- 20 4. The antibody molecule of claim 3, which comprises SEQ ID NO:1 for CDRH1,
SEQ ID NO: 2 or SEQ ID NO:7 for CDRH2, SEQ ID NO:3 for CDRH3, SEQ ID NO:4 for
CDRL1, SEQ ID NO:5 for CDRL2 and SEQ ID NO:6 for CDRL3.
5. The antibody molecule of any one of claims 1 to 4, which comprises SEQ ID NO:2
25 for CDRH2.
6. The antibody molecule of any one of claims 1 to 5, which is a CDR-grafted
antibody molecule.
- 30 7. The antibody molecule of claim 6, wherein the variable domain comprises human
acceptor framework regions and non-human donor CDRs.

8. The antibody molecule of claim 7, wherein the human acceptor framework regions of the variable domain of the heavy chain are based on a human group 1 consensus sequence and comprise non-human donor residues at positions 28, 69 and 71
- 5 9. The antibody molecule of claim 7, wherein the human acceptor framework regions of the variable domain of the heavy chain are based on a human group 1 consensus sequence and comprise non-human donor residues at positions 28, 38, 46, 67, 69 and 71.
- 10 10. The antibody molecule of claim 7, wherein the human acceptor framework regions of the variable domain of the heavy chain are based on a human group 3 consensus sequence and comprise non-human donor residues at positions 27, 28, 30, 48 49, 69, 71, 73 76 and 78.
- 15 11. The antibody molecule of any one of claims 7 to 10, wherein the human acceptor framework regions of the variable domain of the light chain are based on human group 1 consensus sequence and comprise non-human donor residues at positions 46 and 60.
- 20 12. The antibody molecule of any one of claims 1 to 11, comprising the light chain variable region hTNF40-gL1 (SEQ ID NO:8) and the heavy chain variable region gh3hTNF40.4 (SEQ ID NO:11).
13. The antibody molecule of any one of claims 1 to 12 which is a Fab fragment.
- 25 14. The antibody molecule of claims 12 and 13, which is a Fab fragment comprising a heavy chain having the sequence given in SEQ ID NO:111 and a light chain having the sequence given in SEQ ID NO:113.
- 30 15. The antibody molecule of any one of claims 1 to 12, which is a modified Fab fragment having at the C-terminal end of its heavy chain one or more amino acids to allow attachment of an effector or reporter molecule.

16. The antibody molecule of claim 15, wherein the additional amino acids form a modified hinge region containing one or two cysteine residues to which the effector or reporter molecule may be attached.
- 5 17. The antibody molecule of claim 12, which is a modified Fab fragment comprising a heavy chain having the sequence given in SEQ ID NO:115 and a light chain having the sequence given in SEQ ID NO:113.
18. An antibody molecule having specificity for human TNF α , having a light chain
10 comprising the sequence given in SEQ ID NO:113.
19. An antibody molecule having specificity for human TNF α , having a light chain consisting of the sequence given in SEQ ID NO:113.
- 15 20. An antibody molecule having specificity for human TNF α , having a heavy chain comprising the sequence given in SEQ ID NO:115.
21. An antibody molecule having specificity for human TNF α , having a heavy chain consisting of the sequence given in SEQ ID NO:115.
- 20 22. An antibody molecule having specificity for human TNF α , having a light chain comprising the sequence given in SEQ ID NO:113 and a heavy chain comprising the sequence given in SEQ ID NO:115.
- 25 23. An antibody molecule having specificity for human TNF α , having a light chain consisting of the sequence given in SEQ ID NO:113 and a heavy chain consisting of the sequence given in SEQ ID NO:115.
24. A variant of the antibody molecule of any one of claims 1 to 23, which has an
30 improved affinity for TNF α .
25. The variant of claim 24 which is obtained by an affinity maturation protocol.

26. The antibody of any one of claims 1 to 3 which is murine anti-TNF α monoclonal antibody hTNF40.
27. The antibody molecule of any one of claims 1 to 3, which is a chimeric antibody
5 molecule comprising the light and heavy chain variable domains of the monoclonal antibody of claim 26.
28. A compound comprising the antibody molecule of any one of claims 15 to 23
10 having covalently attached to an amino acid at or towards the C-terminal end of its heavy chain an effector or reporter molecule.
29. The compound of claim 28, which comprises an effector molecule.
30. The compound of claim 29, wherein the effector molecule comprises one or more
15 polymers.
31. The compound of claim 30, wherein the one or more polymers is/are an optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymer or a branched or unbranched polysaccharide.
20
32. The compound of claim 31, wherein the one or more polymers is/are a methoxypoly(ethyleneglycol).
33. A compound comprising the antibody molecule of claim 17 having attached to one
25 of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.
34. A compound comprising an antibody molecule having specificity for human TNF α ,
30 having a light chain comprising the sequence given in SEQ ID NO:113 and a heavy chain comprising the sequence given in SEQ ID NO:115, having attached to one of the cysteine residues at the C-terminal end of the heavy chain one or more synthetic or naturally-occurring polymers.

35. A compound comprising an antibody molecule having specificity for human TNF α , having a light chain consisting of the sequence given in SEQ ID NO:113 and a heavy chain consisting of the sequence given in SEQ ID NO:115, having attached to one of the cysteine residues at the C-terminal end of the heavy chain one or more synthetic or naturally-occurring polymers.
36. A compound comprising an antibody molecule having specificity for human TNF α , having a light chain comprising the sequence given in SEQ ID NO:113, having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.
37. A compound comprising an antibody molecule having specificity for human TNF α , having a light chain consisting of the sequence given in SEQ ID NO:113, having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.
38. A compound comprising an antibody molecule having specificity for human TNF α , having a heavy chain comprising the sequence given in SEQ ID NO:115, having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.
39. A compound comprising an antibody molecule having specificity for human TNF α , having a heavy chain consisting of the sequence given in SEQ ID NO:115, having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxy poly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.
40. A compound comprising an antibody molecule having specificity for human TNF α , having a light chain comprising the sequence given in SEQ ID NO:113 and a heavy chain

comprising the sequence given in SEQ ID NO:115, having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.

5

41. A compound comprising an antibody molecule having specificity for human TNF α , having a light chain consisting of the sequence given in SEQ ID NO:113 and a heavy chain consisting of the sequence given in SEQ ID NO:115, having attached to one of the cysteine residues at the C-terminal end of the heavy chain a lysyl-maleimide group wherein each
10 amino group of the lysyl residue has covalently linked to it a methoxypoly(ethyleneglycol) residue having a molecular weight of about 20,000 Da.

42. An antibody molecule comprising a hybrid CDR comprising a truncated donor CDR sequence wherein the missing portion of the donor CDR is replaced by a different sequence
15 and forms a functional CDR.

43. The antibody molecule of claim 42, wherein the missing part of the CDR sequence is from the antibody from which the framework regions of the antibody molecule are derived.

20

44. The antibody molecule of claim 43, wherein the missing part of the CDR sequence is derived from a germline antibody having consensus framework regions.

45. The antibody molecule of any one of claims 42 to 44, wherein CDRH2 of the heavy
25 chain is hybrid in the antibody molecule.

46. The antibody molecule of any one of claims 42 to 45, wherein the truncation of the donor CDR is from 1 to 8 amino acids.

30 47. The antibody molecule of claim 46, wherein the truncation is from 4 to 6 amino acids.

48. The antibody molecule of any one of claims 42 to 47, wherein the truncation is made at the C-terminus of the CDR.
49. A DNA sequence which encodes the heavy and/or light chain of the antibody molecule of any one of claims 1 to 27 and 42 to 48.
50. The DNA sequence of claim 49 comprising the sequence shown in SEQ ID NO:8 or 10.
51. The DNA sequence of claim 49 comprising the sequence shown in SEQ ID NO:10 or 11.
52. The DNA sequence of claim 49 comprising the sequence shown in SEQ ID NO:110, 112 or 114.
53. A cloning or expression vector containing the DNA sequence of any one of claims 49 to 52.
54. An *E. coli* expression vector comprising the DNA sequence of any one of claims 49 to 52.
55. The *E. coli* expression vector of claim 54 which is pTTO(CDP870).
56. A host cell transformed with the vector of any one of claims 53 to 55.
57. A process for the production of the antibody molecule of any one of claims 1 to 27 and 42 to 48, comprising culturing the host cell of claim 56 and isolating the antibody molecule.
58. A process for the production of the antibody molecule of any one of claims 1 to 27 and 42 to 48, comprising culturing *E. coli* comprising an *E. coli* expression vector comprising the DNA sequence of any one of claims 53 to 55 and isolating the antibody molecule.

59. The process of claim 58 wherein the antibody molecule is targeted to the periplasm.
60. A therapeutic or diagnostic composition comprising the antibody molecule of any
5 one of claims 1 to 27 and 42 to 48 or the compound of any one of claims 28 to 41.
61. The antibody molecule of any one of claims 1 to 27 and 42 to 48, having specificity
for human TNF α , or the compound of any one of claims 28 to 41, for use in treating a
pathology mediated by TNF α .
- 10 62. The antibody molecule of or compound claim 61, for use in treating rheumatoid- or
osteo- arthritis.
63. Use of the antibody molecule of any one of claims 1 to 27 and 42 to 48, having
15 specificity for human TNF α , or the compound of any one of claims 28 to 41 in the
manufacture of a medicament for the treatment of a pathology mediated by TNF α .
64. The use of claim 63, wherein the pathology is rheumatoid- or osteo- arthritis.
- 20 65. The vector pDNAEng-G1 as shown in Figure 19.
66. The vector pTTO(CDP870) as shown in Figure 22.
67. A polypeptide having the amino acid sequence given in any one of SEQ ID NOS:1
25 to 7.

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FIG. 1

Comparisons of framework regions of light chain of
antibody hTNF40 and human group 1 consensus sequences

Hu group 1 consensus : DIQMTQSPSSLSASVGDRVTITC (SEQ ID NO: 83)
hTNF40 : DIVMTQSOKFMSTSVGDRVSVTC (SEQ ID NO: 84)

Hu Group 1 consensus : WYQQKPGKAPKLLIY (SEQ ID NO: 85)
hTNF40 : WYQQKPGOSPKALIY (SEQ ID NO: 86)

Hu Group 1 consensus : GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC (SEQ ID NO: 87)
hTNF40 : GVPYRFTGSGSGTDFTLTISTVQSEDLAEYFC (SEQ ID NO: 88)

Hu Group 1 consensus : FGQGTKVEIKR (SEQ ID NO: 89)
hTNF40 : FGAGTKLELKR (SEQ ID NO: 90)

FIG. 3 Sequence of CDRs of hTNF40

H1 DYGMN (SEQ ID NO:1)

H2 WINTYIGEPIYVDDFKG (SEQ ID NO:7)

H2' WINTYIGEPIYADSVKG (SEQ ID NO:2)

H3 GYRSYAMDY (SEQ ID NO:3)

L1 KASQNVGTNVA (SEQ ID NO:4)

L2 SASFLYS (SEQ ID NO:5)

L3 QQYNIYPLT (SEQ ID NO:6)

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FIG. 2

Comparisons of framework regions of heavy chain of antibody hTNF40 and human group 1 and group 3 consensus sequences

Hu Group 1 consensus	: QVQLVQSGAEVKKPGASVKVSCKASGYTFT	(SEQ ID NO: 91)
hTNF40	: <u>Q</u> IQLVQSG <u>P</u> ELKKP <u>G</u> ETVKISCKASGY <u>V</u> FT	(SEQ ID NO: 92)
Hu Group 1 consensus	: WVRQAPGQGLEWMG	(SEQ ID NO: 93)
hTNF40	: WVKQAPGKAFKWMG	(SEQ ID NO: 94)
Hu Group 1 consensus	: RVTITRDTSTSTAYMELSSLRSEDVAVYYCAR	(SEQ ID NO: 95)
hTNF40	: <u>R</u> FAFSLETS <u>A</u> STAF <u>L</u> QINN <u>L</u> KNE <u>D</u> TATY <u>F</u> CAR	(SEQ ID NO: 96)
Hu Group 1 consensus	: WGQGTTLTVSS	(SEQ ID NO: 97)
hTNF40	: WGQGT <u>T</u> TLTVSS	(SEQ ID NO: 98)
Hu Group 3 consensus	: EVQLVESGGGLVQPGGSLRLSCAASGFTFS	(SEQ ID NO: 106)
hTNF40	: <u>Q</u> IQLVQSG <u>P</u> ELKKP <u>G</u> ETVKISCKASGY <u>V</u> FT	(SEQ ID NO: 92)
Hu Group 3 consensus	: WVRQAPGKGLEWVS	(SEQ ID NO: 107)
hTNF40	: WVKQAPGKAFKWMG	(SEQ ID NO: 94)
Hu Group 3 consensus	: RFTISRDN SKNTLYLQMN SLRAEDTAVYYCAR	(SEQ ID NO: 108)
hTNF40	: <u>R</u> FAFSLETS <u>A</u> STAF <u>L</u> QINN <u>L</u> KNE <u>D</u> TATY <u>F</u> CAR	(SEQ ID NO: 96)
Hu Group 3 consensus	: WGQGTTLTVSS	(SEQ ID NO: 109)
hTNF40	: WGQGT <u>T</u> TLTVSS	(SEQ ID NO: 98)

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FIG. 4

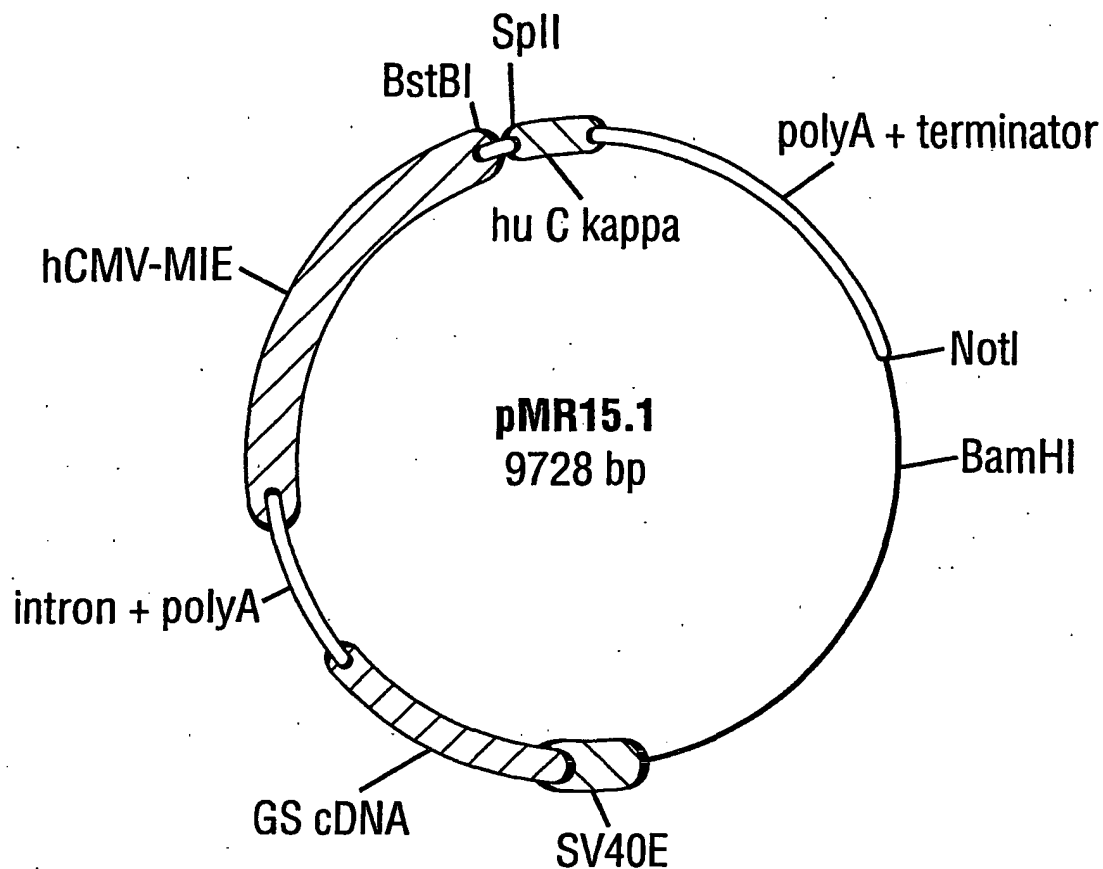
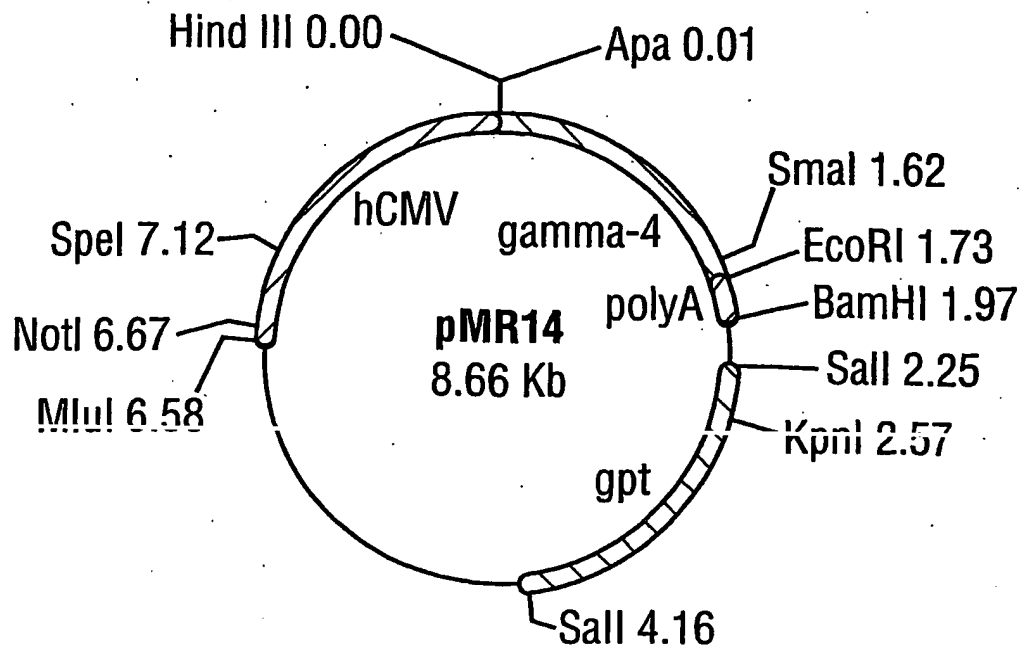


FIG. 5



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FIG. 6 Murine VI Sequence of hTNF40 (SEQ ID NO: 99)

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10  20  30  40  50
GAC ATT GTG ATG ACC CAG TCT CAA AAA TTC ATG TCC ACA TCA GTA GGA GAC AGG
CTG TAA CAC TAC TGG GTC AGA GTT TTT AAG TAC AGG TGT AGT CAT CCT CTG TCC
T  I  V  M  T  Q  S  Q  K  F  M  S  T  S  V  G  D  R>

60  70  80  90  100
GTC AGC GTC ACC TGC AAG GCC AGT CAG AAT GTG GGT ACT AAT GTA GCC TGG TAT
CAG TCG CAG TGG ACG TTC CGG TCA GTC TTA CAC CCA TGA TTA CAT CCG ACC ATA
V  S  V  T  C  K  A  S  Q  N  V  G  T  N  V  A  W  Y>

110 120 130 140 150 160
CFA CAG AAA CCA GGA CAA TCT CCT CCA GGC AGT GGA ATT TAC TCG GCA TCC TTC CTA
GTT GTC TTT GGT CCT GGT GGA TTT CGT GAC TAA ATG AGC CGT AGG AAG GAT
C  Q  K  P  G  Q  S  P  K  A  L  I  Y  S  A  S  F  L>

170 180 190 200 210
TAT AGT GGA GTC CCT TAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT
ATA TCA CCT CAG GGA ATA GCG AAG TGT CCG TCA CCT AGA CCC TGT CTA AAG TGA
V  S  G  V  P  Y  R  F  T  G  S  G  S  G  T  D  F  T>

220 230 240 250 260 270
CCT ACC ATC AGC ACT GTG CAG TCT GAA GAC TTG GCA GAG TAT TTC TGT CAG CAA
GAG TGG TAG TCG TGA CAC GTC AGA CTT CTG AAC CGT CTC ATA AAG ACA GTC GTT
L  T  I  S  T  V  Q  S  E  D  L  A  E  Y  F  C  Q  Q>

280 290 300 310 320
TAT AAC ATC TAT CCT CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGT
ATA TTG TAG ATA GGA GAG TGC AAG CCA CGA CCC TGG TTC GAC CTC GAC TTT GCA
T  N  I  Y  P  L  T  F  G  A  G  T  K  L  E  L  K  R>

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FIG. 7 Murine Vh Sequence of hTNF40 (SEQ ID NO: 100)

```

10      20      30      40      50
CAG ATC CAG TTG GTG CAG TCT GGA CCT AAG AAG CCG GGA GAG ACA GTC
GTC TAG GTC AAC CAC GTC AGA CCT GGA CTC GAC TTC TTC GGA CCT CTC TGT CAG
Q I Q L V Q S G P E L K K P G E T V>

60      70      80      90      100
AAG ATC TCC TGC AAG GCT TCT GGA TAT GGT TTC ACA GAC TAT GGA ATG AAT TGG
TTC TAG AGG ACG TTC CGA AGA CCT ATA CAA AAG TGT CTG ATA CCT TAC TTA ACC
K I S C K A S G Y V F T D Y G M N W>

110     120     130     140     150     160
GTG AAG CAG GCT CCA GGA AAG GCT TTC AAG TGG ATG GGC TGG ATA AAC ACC TAC
CAC TTC GTC CGA GGT CCT TTC CGA AAG TTC ACC TAC CCG ACC TAT TTG TGG ATG
V K Q A P G K A F K W M G W I N T Y>

170     180     190     200     210
ATT GGA GAG CCA ATA TAT GTT GAT GAC TTC AAG GGA CGA TTT GCC TTC TCT TTG
TAA CCT CTC GGT TAT ATA CAA CTA CTG AAG TTC CCT GCT AAA CGG AAG AGA AAC
I G E P I Y V D D F K G R F A F S L>

220     230     240     250     260     270
GAA ACC TCT GCC AGC ACT GCC TTT TTG CAG ATC AAC AAC CTC AAA AAT GAG GAC
CTT TGG AGA CGG TCG TGA CGG AAA AAC AAC GTC TAG TTG TGG GAG TTT TTA CTC CTG
E T S A S T A F L Q I N N L K N E D>

280     290     300     310     320
ACG GCT ACA TAT TTC TGT GCA AGA GGT TAC CGG TCC TAT GCT ATG GAC TAC TGG
TGC CGA TGT ATA AAG ACA CGT TCT CCA ATG GGC AGG ATA CGA TAC CTG ATG ACC
T A T Y F C A R G Y R S Y A M D Y W>

330     340     350
GGT CAA GGA ACC TCA GTC ACC GTC TCT TCA
CCA GTT CCT TGG AGT CAG TGG CAG AGA AGT
G Q G T S V T V S S>

```


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FIG. 8 Grafted VI Sequence of hTNF40 (SEQ ID NO: 8)

```

10  20  30  40  50
GAC ATT CAA ATG ACC CAG AGC CCA TCC AGC CTG AGC GCA TCT GTA GGA GAC CGG
CTG TAA GTT TAC TGG GTC TCG GGT AGG TCG GAC TCG CGT AGA CAT CCT CTG GCC
D  I  Q  M  T  Q  S  P  S  L  S  A  S  V  G  D  R>

60  70  80  90  100
GTC ACC ATC ACT TGT AAA GCC AGT CAG AAC GTA GGT ACT AAC GTA GCC TGG TAT
CAG TGG TAG TGA ACA TTT CGG TCA GTC TTG CAT CCA TGA TTG CAT CGG ACC ATA
V  T  I  T  C  K  A  S  Q  N  V  G  T  N  V  A  W  Y>

110 120 130 140 150 160
CAG CAA AAA CCA GGT AAA GCC CCA AAA GCC CTC ATC TAC AGT GCC TCT TTC CTC
GTC GTT TTT GGT CCA TTT CGG GGT TTT CGG GAG TAG ATG TCA CGG AGA AAG GAG
Q  Q  K  P  G  K  A  P  K  A  L  I  Y  S  A  S  F  L>

170 180 190 200 210
TAT AGT GGT GTA CCA TAC TAC AGG TTC AGC GGA TCC GGT AGT GGT ACT GAT TTC ACC
ATA TCA CCA CAT GGT ATG TCC AAG TCG CCT AGG CCA TCA CCA TGA CTA AAG TGG
Y  S  G  V  P  Y  R  F  S  G  S  G  T  D  F  T>

220 230 240 250 260 270
CTC ACG ATC AGT AGC CTC CAG CCA GAA GAT TTC GCC ACT TAT TAC TGT CAA CAG
GAG TGC TAG TCA TCG GAG GTC GGT CTT CTA AAG CGG TGA ATA ATG ACA GTT GTC
L  T  I  S  S  L  Q  P  E  D  F  A  T  Y  Y  C  Q  Q>

280 290 300 310 320
TAT AAC ATC TAC CCA CTC ACA TTC GGT CAG GGT ACT AAA GTA GAA ATC AAA
ATA TTG TAG ATG GGT GAG TGT AAG CCA GTC CCA TGA TTT CAT CTT TAG TTT
Y  N  I  Y  P  L  T  F  G  Q  G  T  K  V  E  I  K>

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FIG. 9 Grafted VI sequence of hTNF40 (SEQ ID NO: 9)

```

10      20      30      40      50
GAC ATT CAA ATG ACC CAG AGC CCA TCC AGC CTG AGC GCA TCT GTA GGA GAC CGG
CTG TAA GTT TAC TGG GTC TCG GGT AGG TCG GAC TCG CGT AGA CAT CCT CTG GCC
D   I   Q   M   T   Q   S   P   S   L   S   A   S   V   G   D   R>

60      70      80      90      100
GTC ACC ATC ACT TGT AAA GCC AGT CAG AAC GTA GGT ACT AAC GTA GCC TGG TAT
CAG TGG TAG TGA ACA TTT CGG TCA GTC TTT CAT CCA TGA TTG CAT CGG ACC ATA
V   T   I   T   C   K   A   S   Q   N   V   G   T   N   V   A   W   Y>

110     120     130     140     150     160
CAG CAA AAA CCA GGT AAA GCC CCA AAA CTC CTC ATC TAC AGT GCC TCT TTC CTC
GTC GTT TTT GGT CCA TTT CGG GGT TTT GAG GAG TAG ATG TCA CGG AGA AAG GAG
Q   Q   K   P   G   K   A   P   K   L   L   I   Y   S   A   S   F   L>

170     180     190     200     210
TAT AGT GGT GTA CCA TAC AGG TTC AGC GGA TCC GGT AGT GGT ACT GAT TTC ACC
ATA TCA CCA CAT GGT ATG TCC AAG TCG CCT AGG CCA TCA CCA TGA CTA AAG TGG
Y   S   G   V   P   Y   R   F   S   G   S   G   S   G   T   D   F   T>

220     230     240     250     260     270
CTC ACG ATC AGT AGC CTC CAG CCA GAA GAT TTC GCC ACT TAT TAC TGT CAA CAG
GAG TGC TAG TCA TCG GAG GTC GGT CTT CTA AAG CGG TGA ATA ATG ACA GTT GTC
L   T   I   S   S   L   Q   P   E   D   F   A   T   Y   Y   C   Q   Q>

280     290     300     310     320
TAT AAC ATC TAC CCA CTC ACA TTC GGT CAG GGT ACT AAA GTA GAA ATC AAA
ATA TTG TAG ATG GGT GAG TGT AAG CCA GTC CCA TGA TTT CAT CTT TAG TTT
Y   N   I   Y   P   L   T   F   G   Q   G   T   K   V   E   I   K>

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FIG. 10 Grafted Vh sequence of hTNF40 (SEQ ID NO: 10)

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10 20 30 40 50
CAG GTG CAG CTG GTC CAG TCA GGA GCA GAG GTT AAG AAG CCT GGT TCC GTC
GTC CAC GTC GAC CAG GTC AGT CCT CGT CTC CAA TTC TTC GGA CCA CGA AGG CAG
Q V Q L V Q S G A E V K K P G A S V>

60 70 80 90 100
AAA GTT TCG TGT AAG GCC TCA GGC TAC GTG TTC ACA GAC TAT GGT ATG AAT TGG
TTT CAA AGC ACA TTC CGG AGT CCG ATG CAC AAG TGT CTG ATA CCA TAC TTA ACC
K V S C K A S G Y V F T D Y G M N W>

110 120 130 140 150 160
GTC AGA CAG GCC CCG GGA CAA GGC CTG GAA TGG ATG GGT TGG ATT AAT ACT TAC
CAG TCT GTC CGG GGC CCT GTT CCG GAC CTT ACC TAC CCA ACC TAA TTA TGA ATG
V R Q A P G Q G L E W M G W I N T Y>

170 180 190 200 210
ATT GGA GAG CCT ATT TAT GCT CAA AAG TTC CAG GGC AGA GTC ACG TTC ACT CTA
TAA CCT CTC GGA TAA ATA CGA GTT TTC AAG GTC CCG TCT CAG TGC AAG TGA GAT
I G E P I Y A Q K F Q G R V T F T L>

220 230 240 250 260 270
GAC ACC TCC ACA AGC ACT GCA TAC ATG GAG CTG TCA TCT CTG AGA TCC GAG GAC
CTG TGG AGG TGT TCG TGA CGT ATG TAC CTC GAC AGT AGA GAC TCT AGG CTC CTG
D T S T S T A Y M E L S L R S E D>

280 290 300 310 320
ACC GCA GTG TAC TAT TGT GCT AGA GGA TAC AGA TCT TAT GCC ATG GAC TAC TGG
TGG CGT CAC ATG ATA ACA CGA TCT CCT ATG TCT AGA ATA CGG TAC CTG ATG ACC
T A V Y Y C A R G Y R S Y A M D Y W>

330 340 350
GGC CAG GGT ACC CTA GTC ACA GTC TCC TCA
CCG GTC CCA TGG GAT CAG TGT CAG AGG AGT
G Q G T L V T V S S>

```

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FIG. 11 Grafted Vh Sequence of hTNF40.4 (SEQ ID NO: 11)

```

10      20      30      40      50
GAG GTT CAG CTG GTC GAG TCA GGA GGC GGT CTC GTG CAG CCT GGC GGA TCA CTG
CTC CAA GTC GAC CAG CAG CTC AGT CCT CCG CCA GAG CAC GTC GGA CCG CCT AGT GAC
E V Q L V V E S G G G L V Q P G G S L>

60      70      80      90      100
AGA TTG TCC TGT GCT GCA TCT GGT TAC GTC TTC ACA GAC TAT GGA ATG AAT TGG
TCT AAC AGG ACA CGA CGT AGA CCA ATG CAG AAG TGT CTG ATA CCT TAC TTA ACC
R L S C A A S G Y V F T D Y G M N W>

110     120     130     140     150     160
GTT AGA CAG GCC CCG GGA AAG GGC CTG GAA TGG ATG GGT TGG ATT AAT ACT TAC
CAA TCT GTC CGG GGC CCT TTC CCG GAC CTT ACC TAC CCA ACC TAA TTA TGA ATG
V R Q A P G K G L E W M G W I N T Y>

170     180     190     200     210
ATT GGA GAG CCT ATT TAT GCT GAC AGC GTC AAG GGC AAT AGC TTC TCT TCT CTA
TAA CCT CTC GGA TAA ATA CGA CTG TCG CAG TTC CCG TCT AAG TGC AAG AGA GAT
I G E P I Y A D S V K G R F T F S L>

220     230     240     250     260     270
GAC ACA TCC AAG TCA ACA GCA TAC CTC CAA ATG AAT AGC CTG AGA GCA GAG GAC
CTG TGT AGG TTC AGT TGT CGT ATG GAG GGT TAC TTA TCG GAC TCT CGT CTC CTG
D T S K S T A Y L Q M N S L R A E D>

280     290     300     310     320
ACC GCA GTG TAC TAT TGT GCT AGA GGA TAC AGA TCT TAT GCC ATG GAC TAC TGG
TGG CGT CAC ATG ATA ACA CGA TCT CCT ATG TCT AGA ATA CGG TAC CTG ATG ACC
T A V Y Y C A R G Y R S Y A M D Y W>

330     340     350
GGC CAG GGT ACC CTA GTC ACA GTC TCC TCA
CCG GTC CCA TGG GAT CAG TGT CAG AGG AGT
G Q G T L V T V S S>

```

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FIG. 12

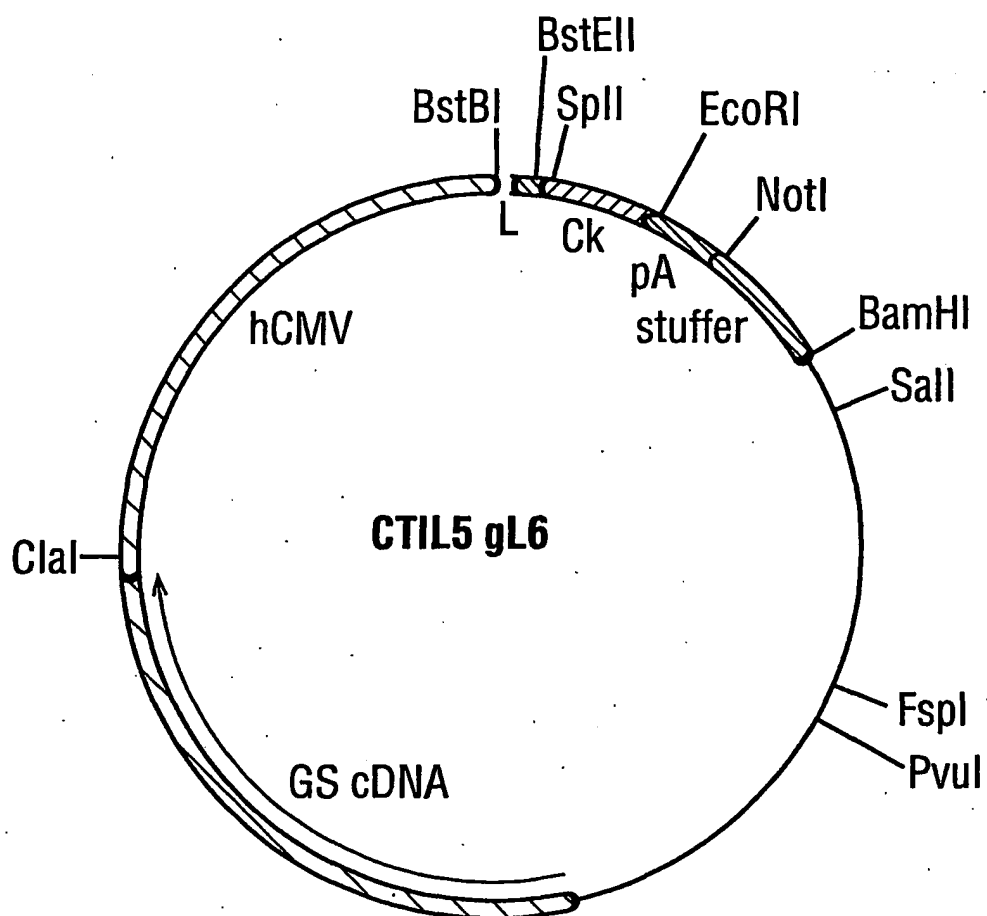
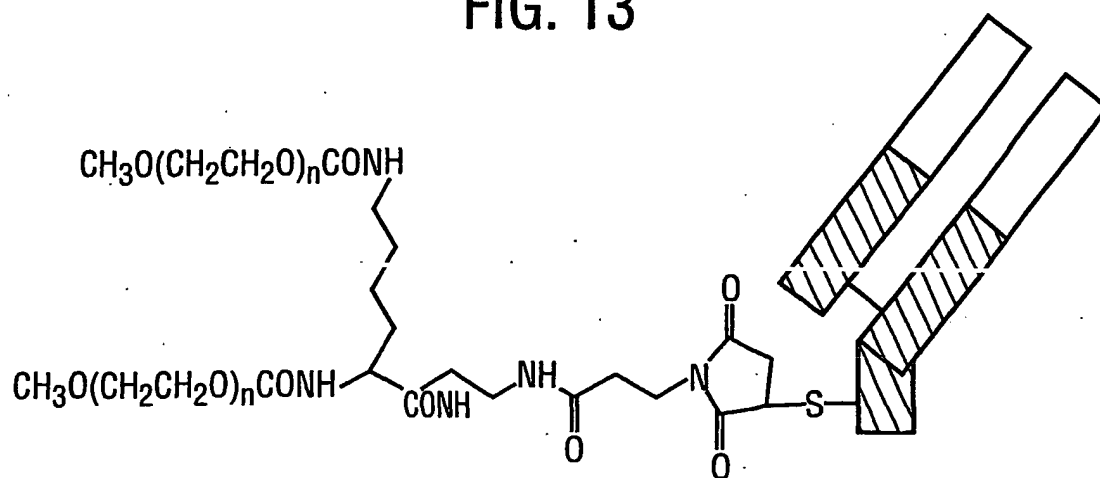
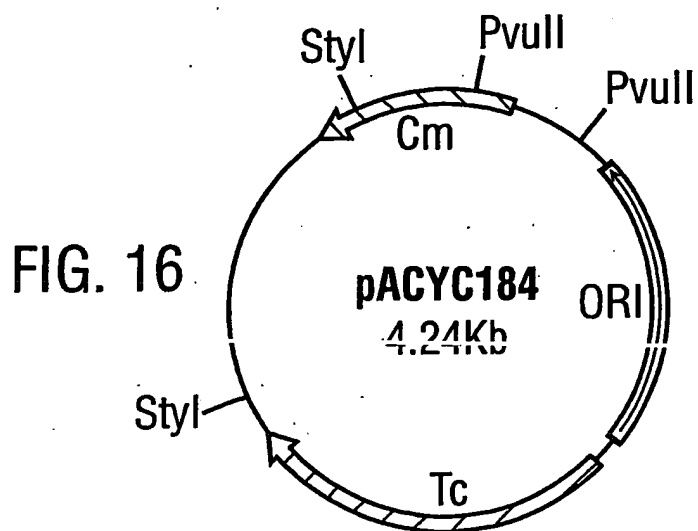
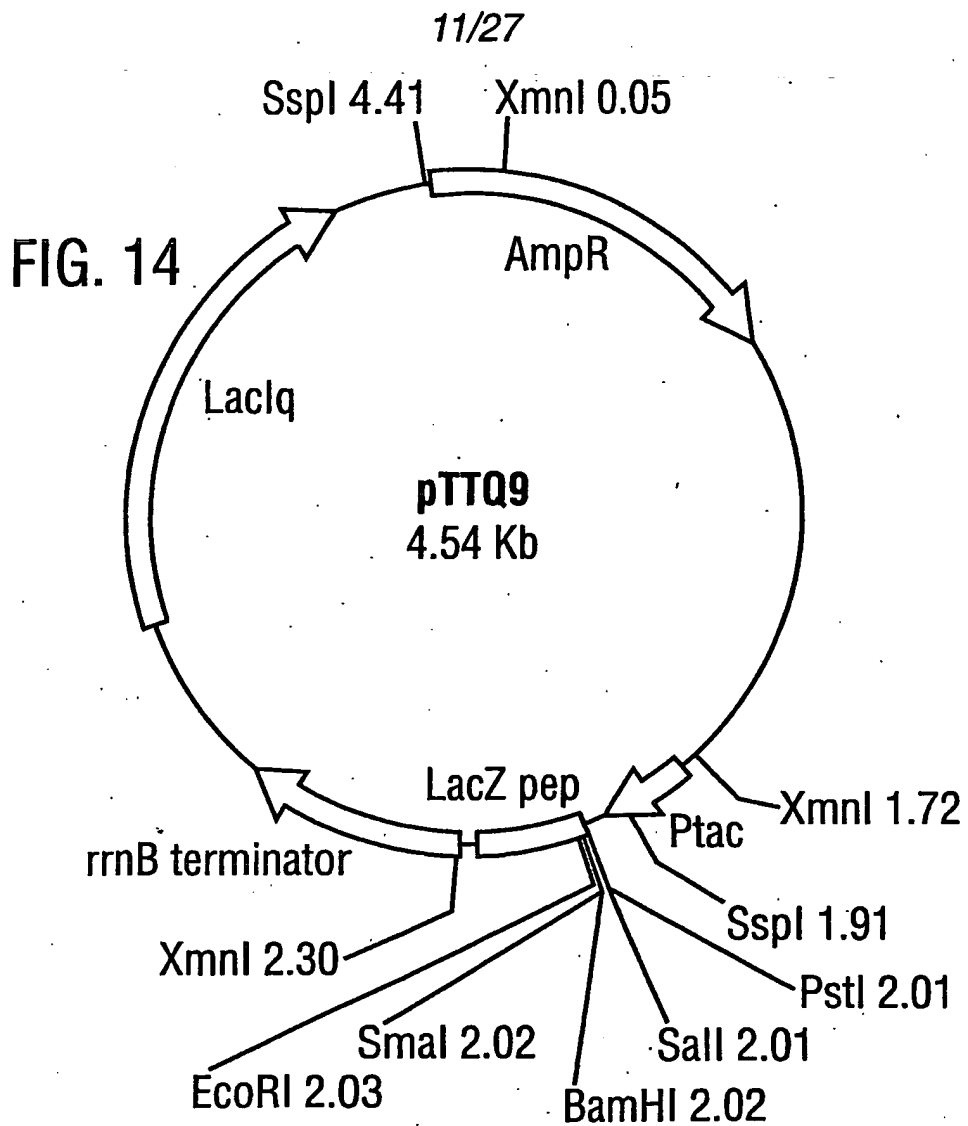


FIG. 13

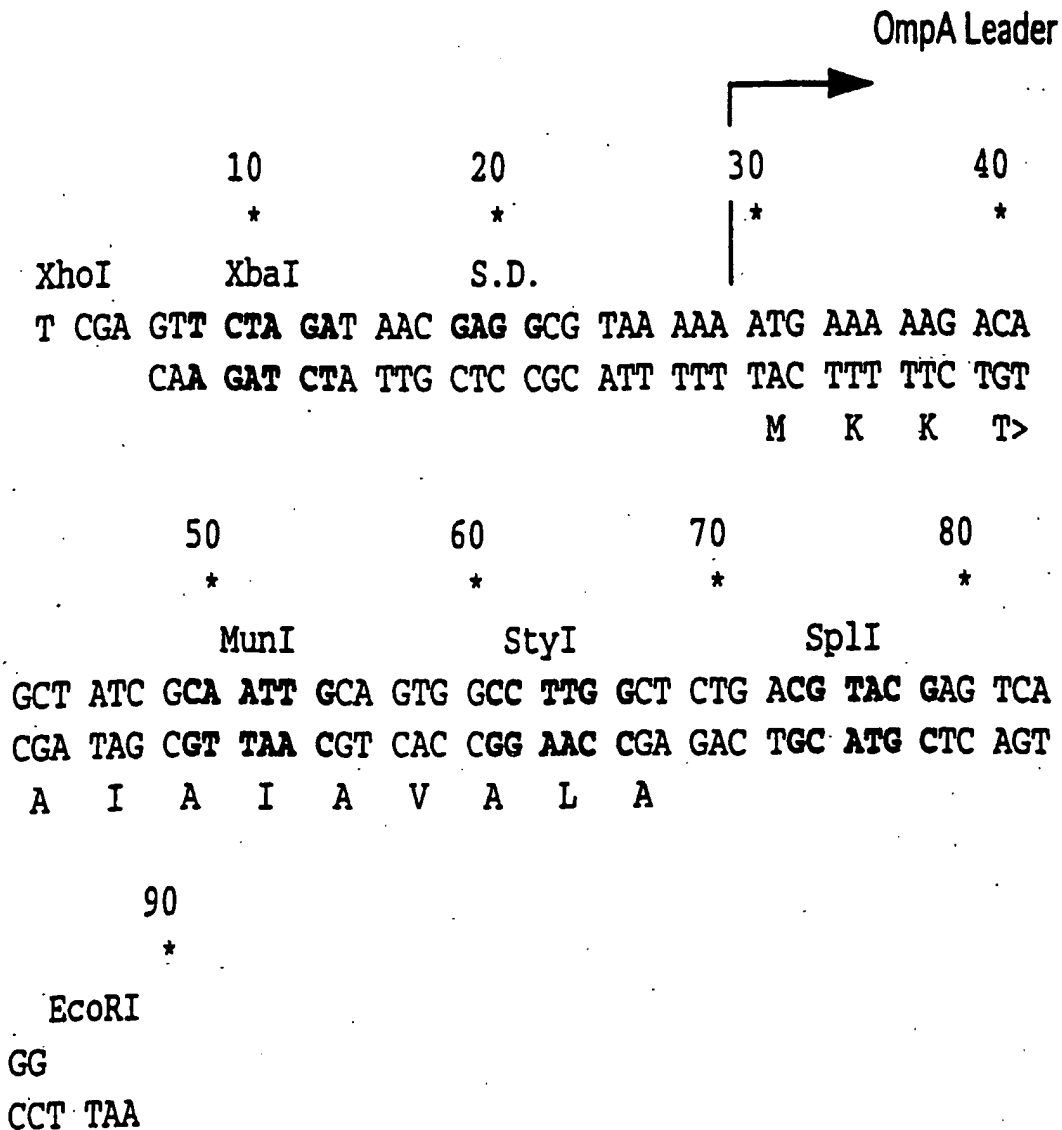




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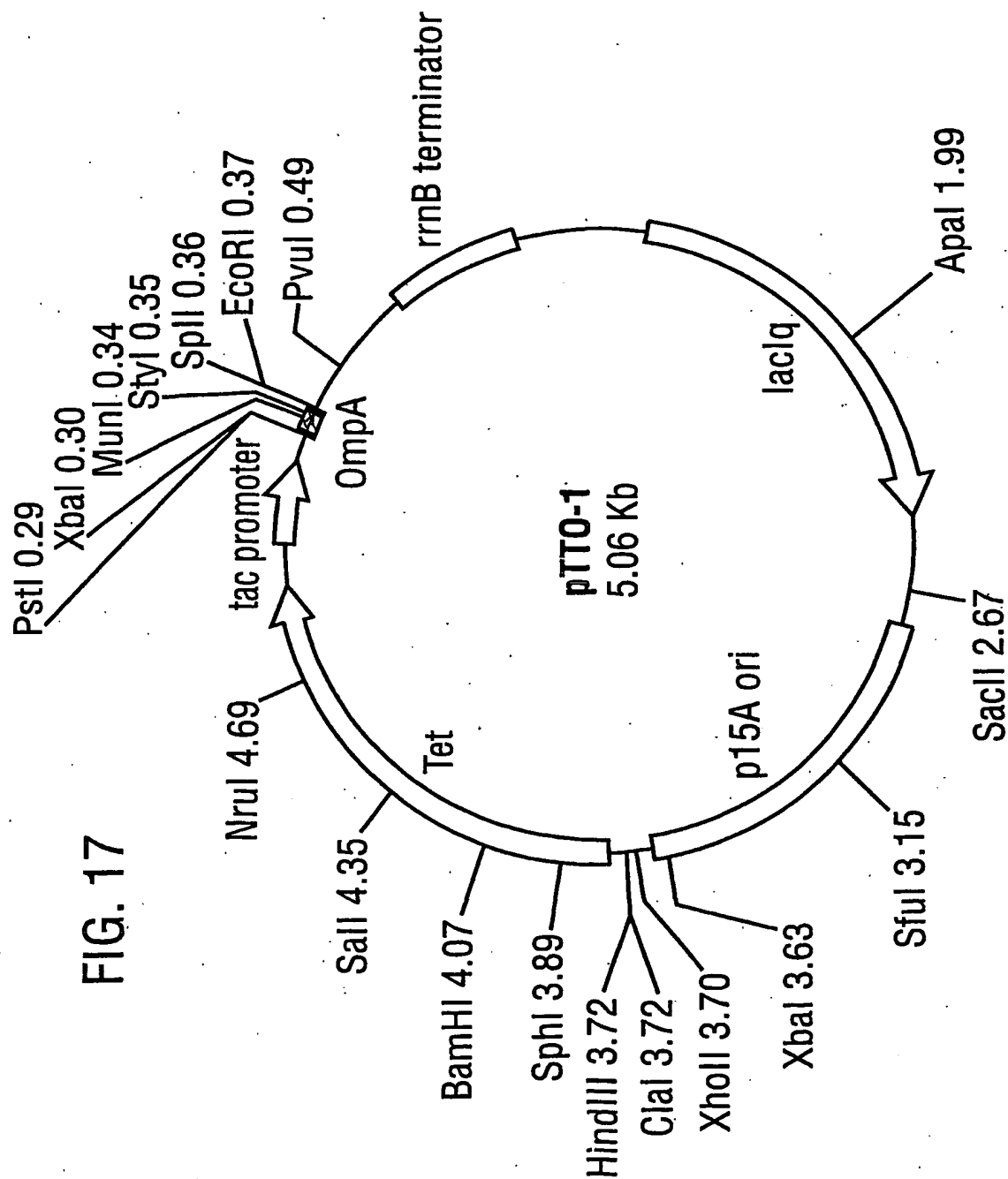
FIG. 15

Sequence of OmpA Oligonucleotide Adapter (SEQ ID NO: 101)

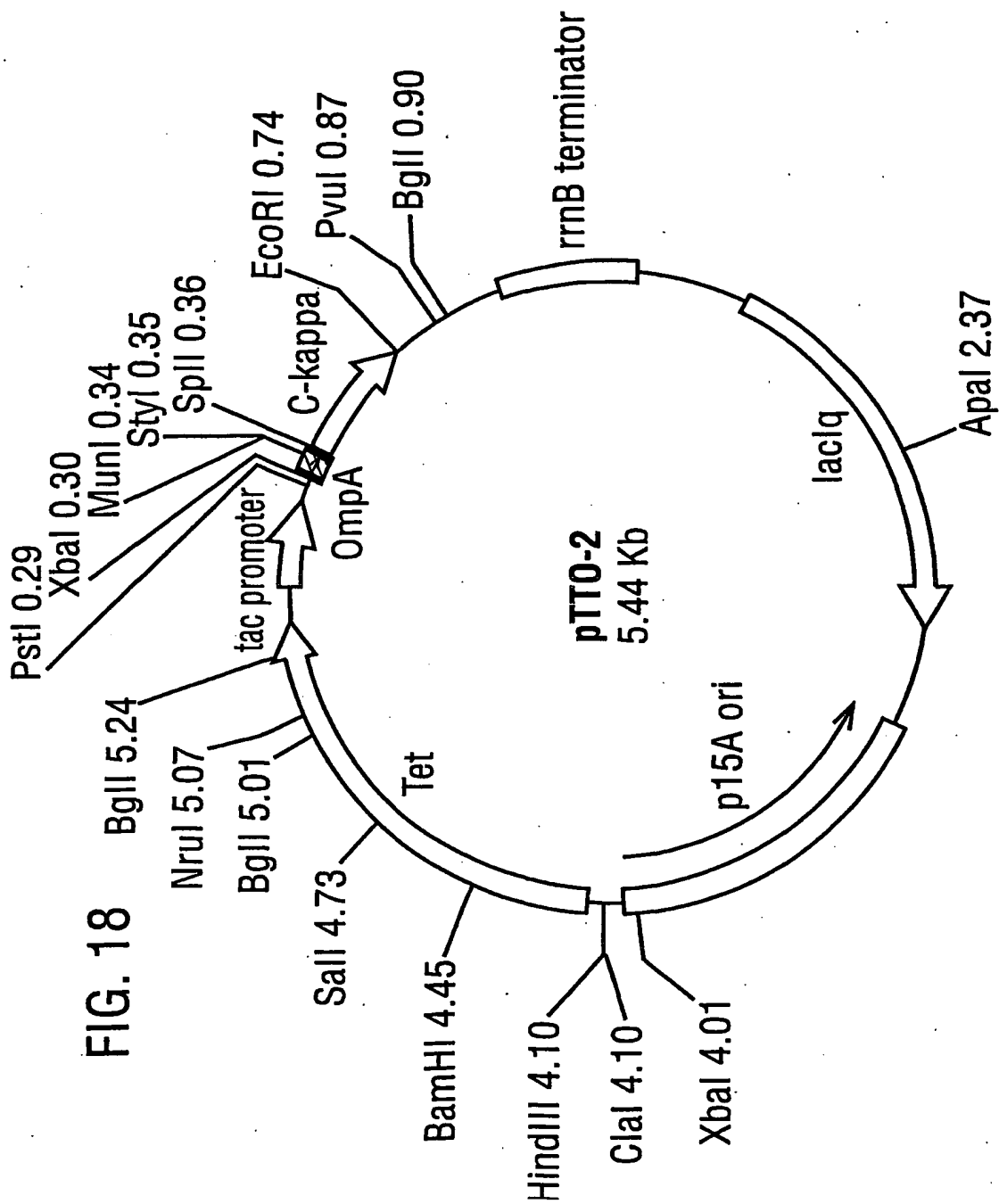


- Internal restriction sites are shown in bold
- The 5' XhoI cohesive end ligates into the Vector Sall site, blocking it
- S.D. represents the OmpA Shine Dalgarno sequence

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FIG. 19

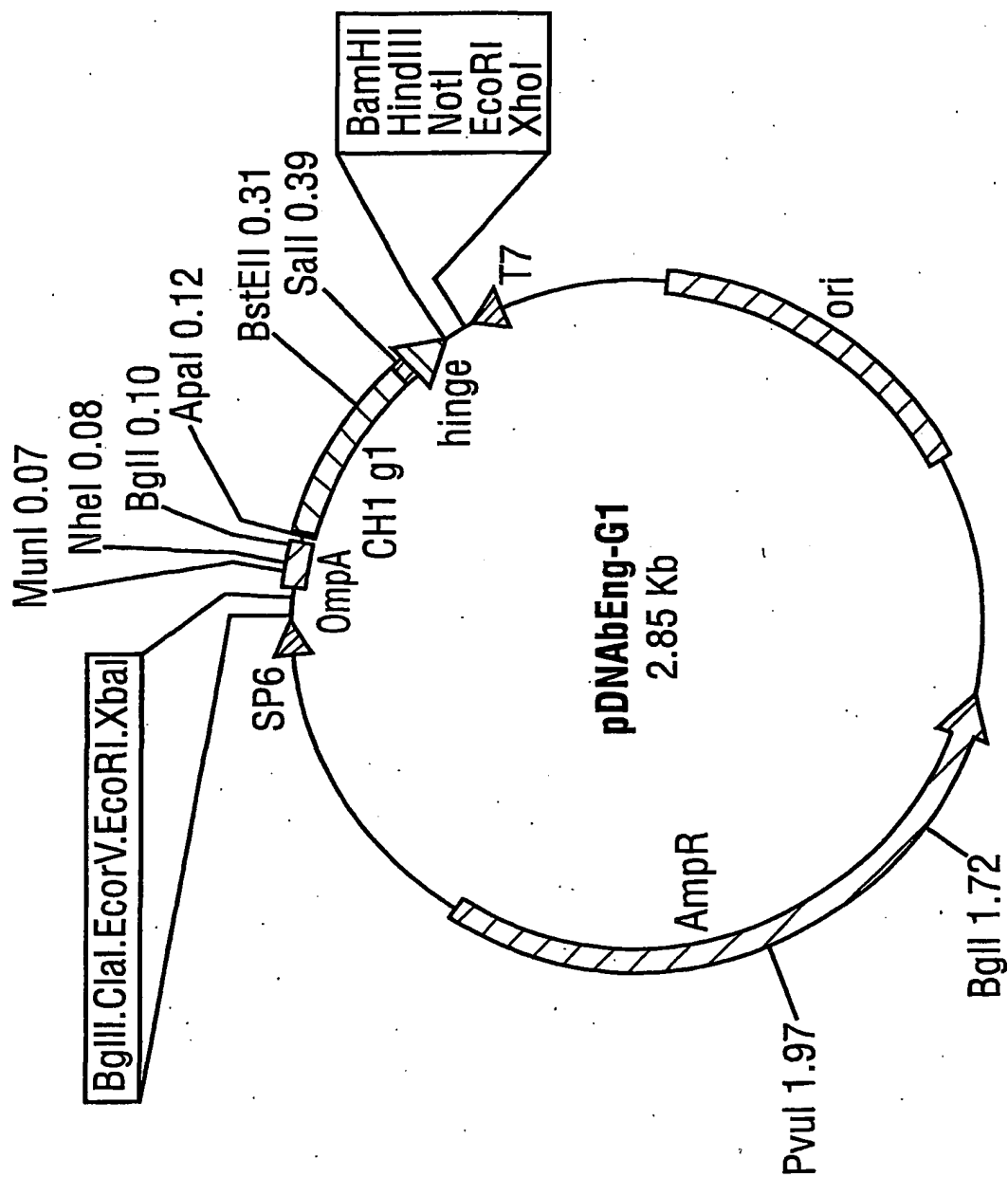


FIG. 20 OLIGONUCLEOTIDE CASSETTES ENCODING DIFFERENT INTERGENIC SEQUENCES FOR E. Coli Fab' EXPRESSION

IGS CASSETTE-1; Intergenic space = -1

G, AGC, TCA, CCA, GTA, ACA, AAA, AGT, TTT, AAT, AGA, GGA, GAG, TGT, TAATG, AAG, AAG, ACT, GCT, ATA, GCA, ATT, G (SEQ ID No: 102)

S S P V T K S F N R G E C * M K K T A I A I

End of c-Kappa sequence ->

Start of OmpA sequence ->

IGS CASSETTE-2; Intergenic space = +1

G, AGC, TCA, CCA, GTA, ACA, AAA, AGT, TTT, AAT, AGA, GGG, GAG, TGT, TAA AATG, AAG, AAG, ACT, GCT, ATA, GCA, ATT, G (SEQ ID No: 103)

S S P V T K S F N R G E C * M K K T A I A I

IGS CASSETTE-3; Intergenic space = +13

G, AGC, TCA, CCA, GTA, ACA, AAA, AGC, TTT, AAT, AGA, GGA, GAG, TGT, TGA GGAGGAAAAAAAATG, AAG, AAA, ACT, GCT, ATA, GCA, ATT, G (SEQ ID No: 104)

S S P V T K S F N R G E C * M K K T A I A I

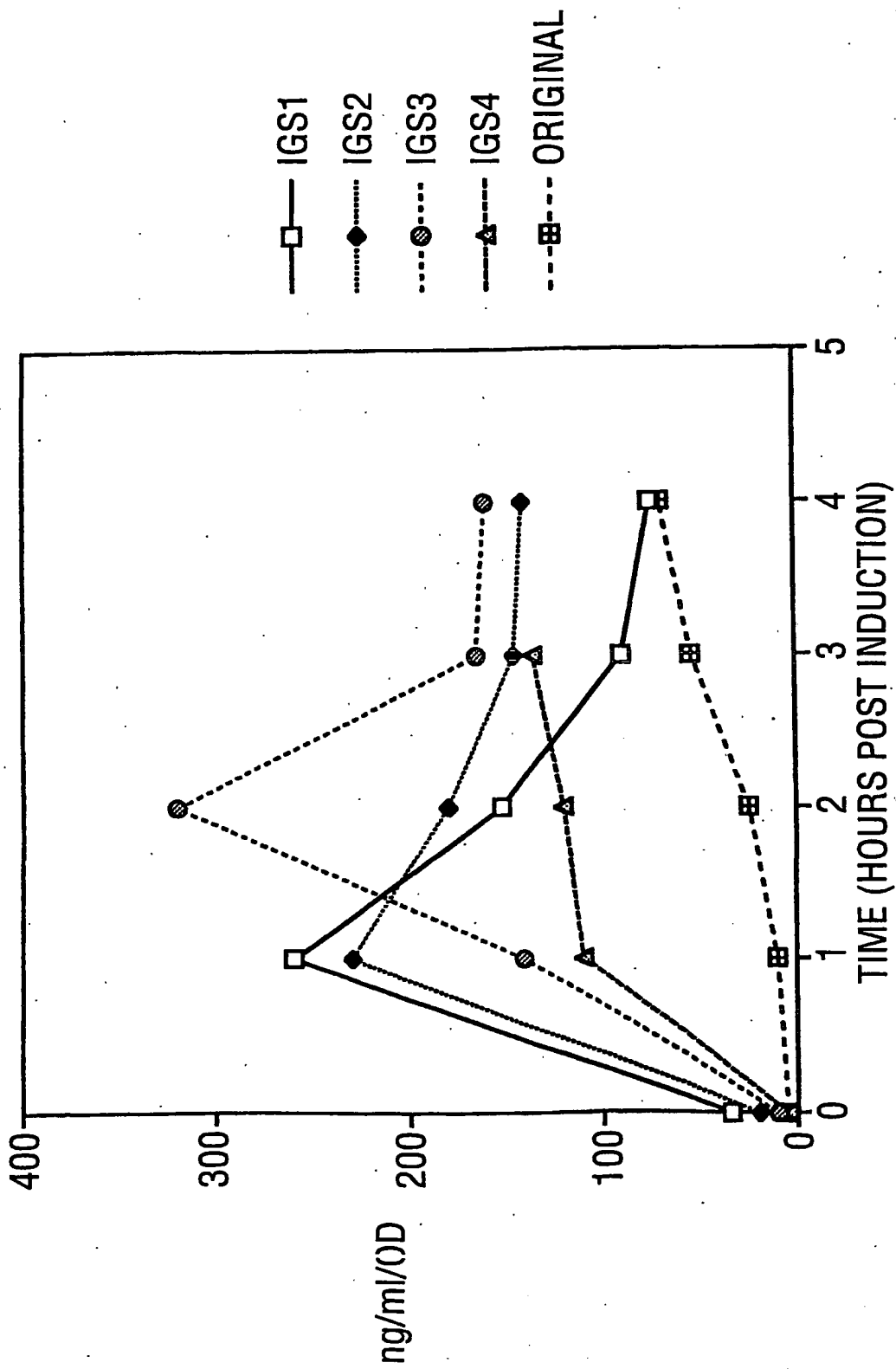
IGS CASSETTE-4; Intergenic space = +13

G, AGC, TCA, CCA, GTA, ACA, AAA, AGT, TTT, AAT, AGA, GGA, GAG, TGT, TGA CGAGGATTATATAATG, AAG, AAA, ACT, GCT, ATA, GCA, ATT, G (SEQ ID No: 105)

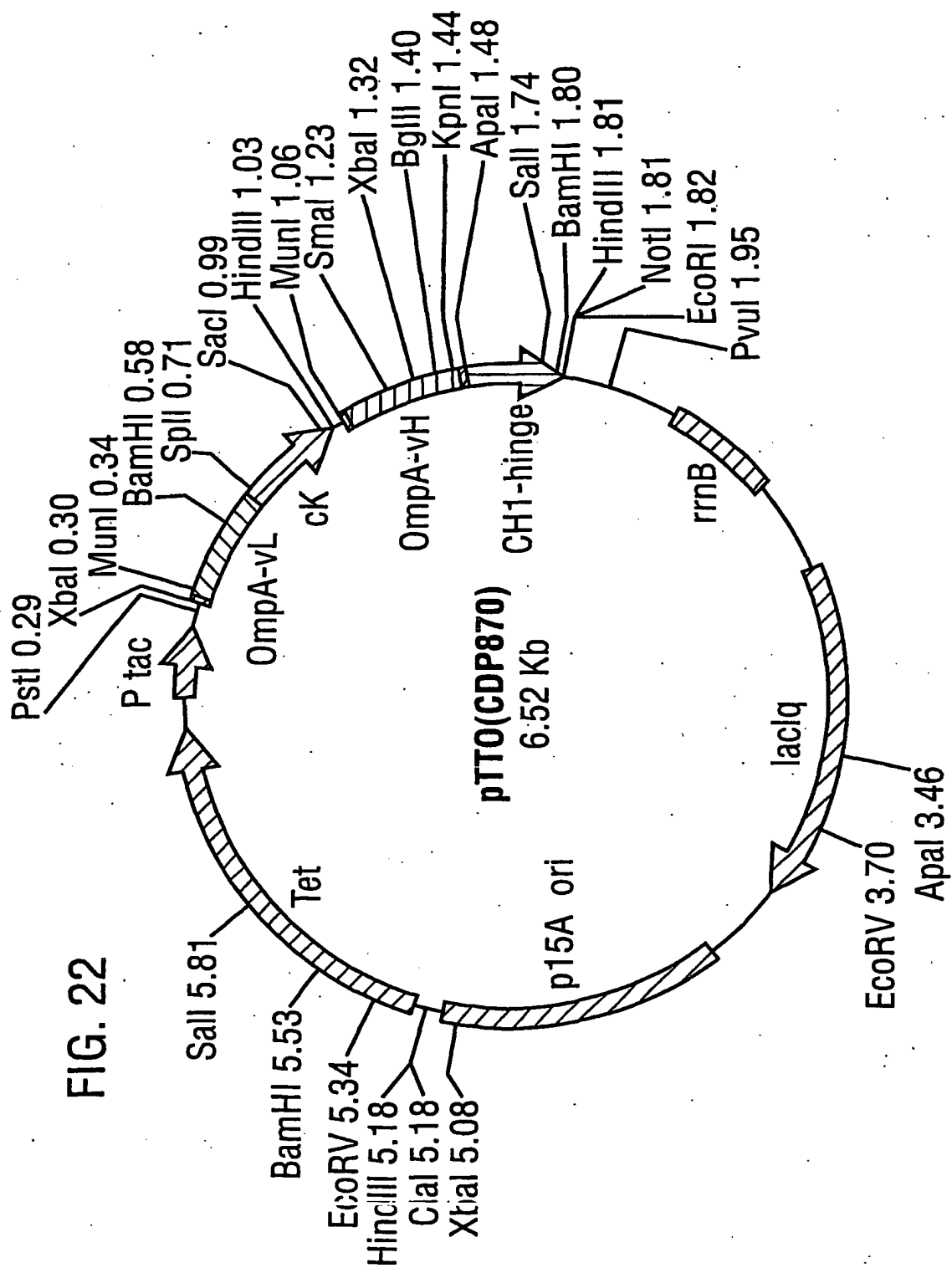
S S P V T K S F N R G E C * M K K T A I A I

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FIG. 21 Periplasmic Fab' accumulation - IGS variants

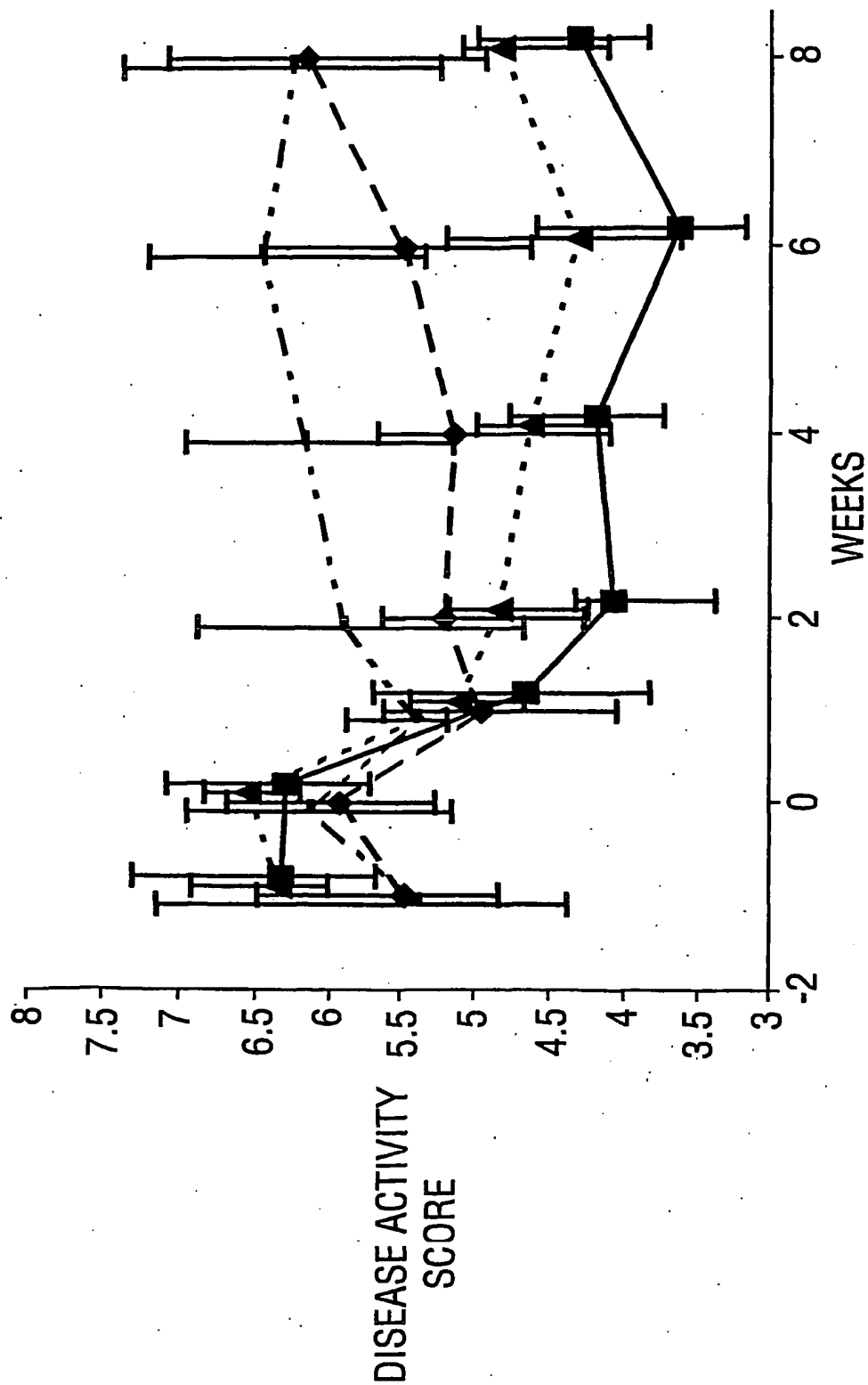


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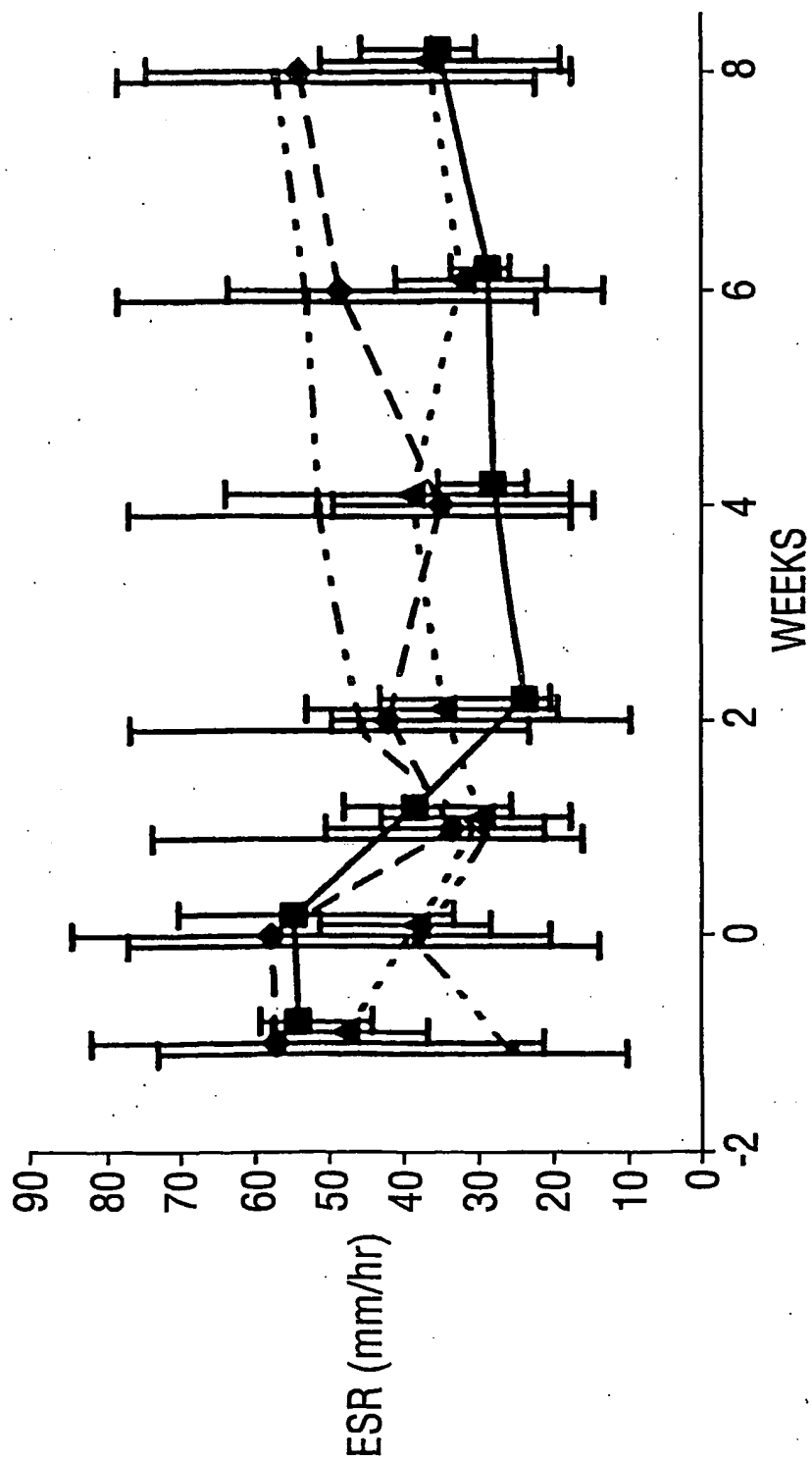
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FIG. 23



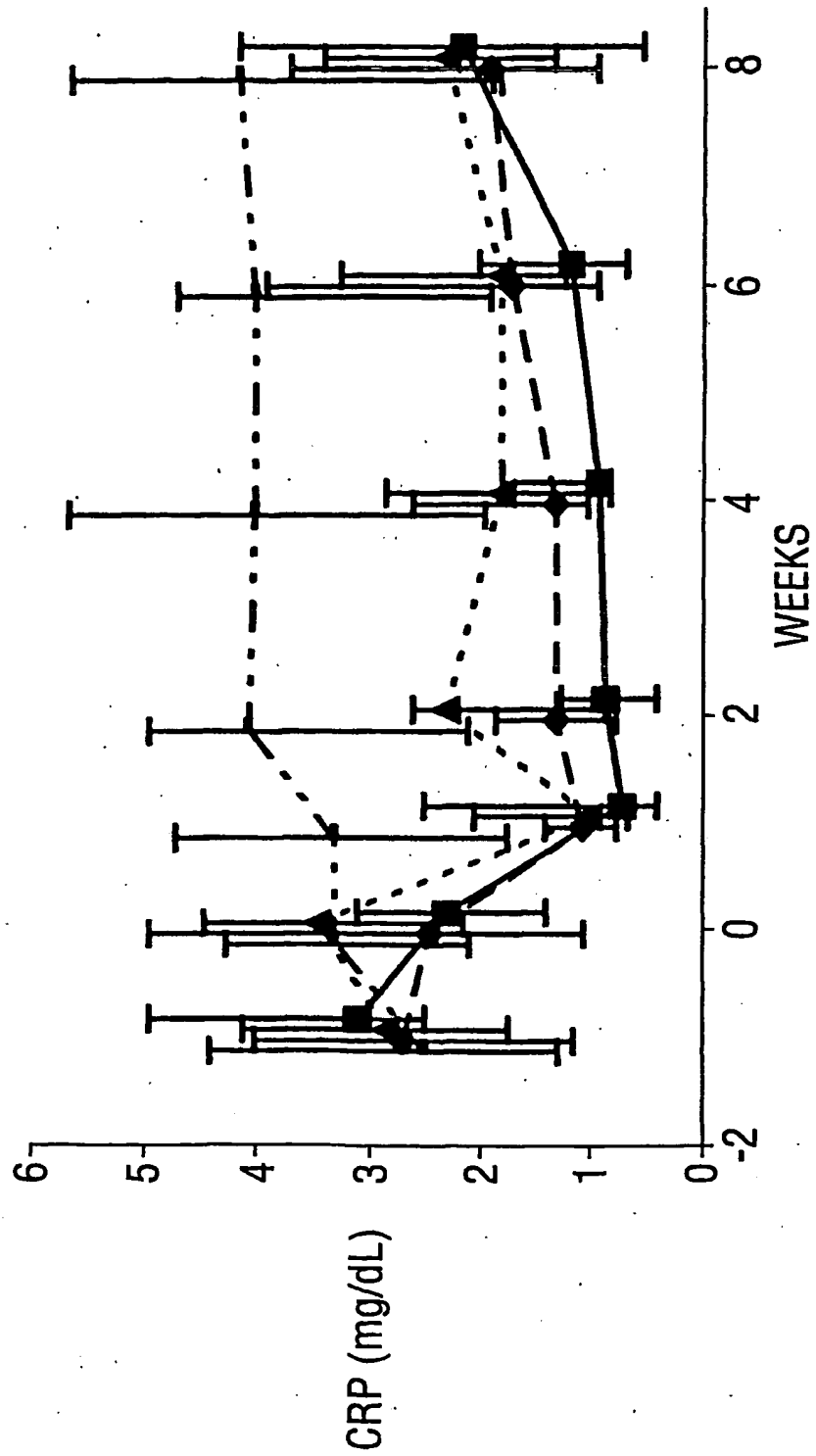
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FIG. 24



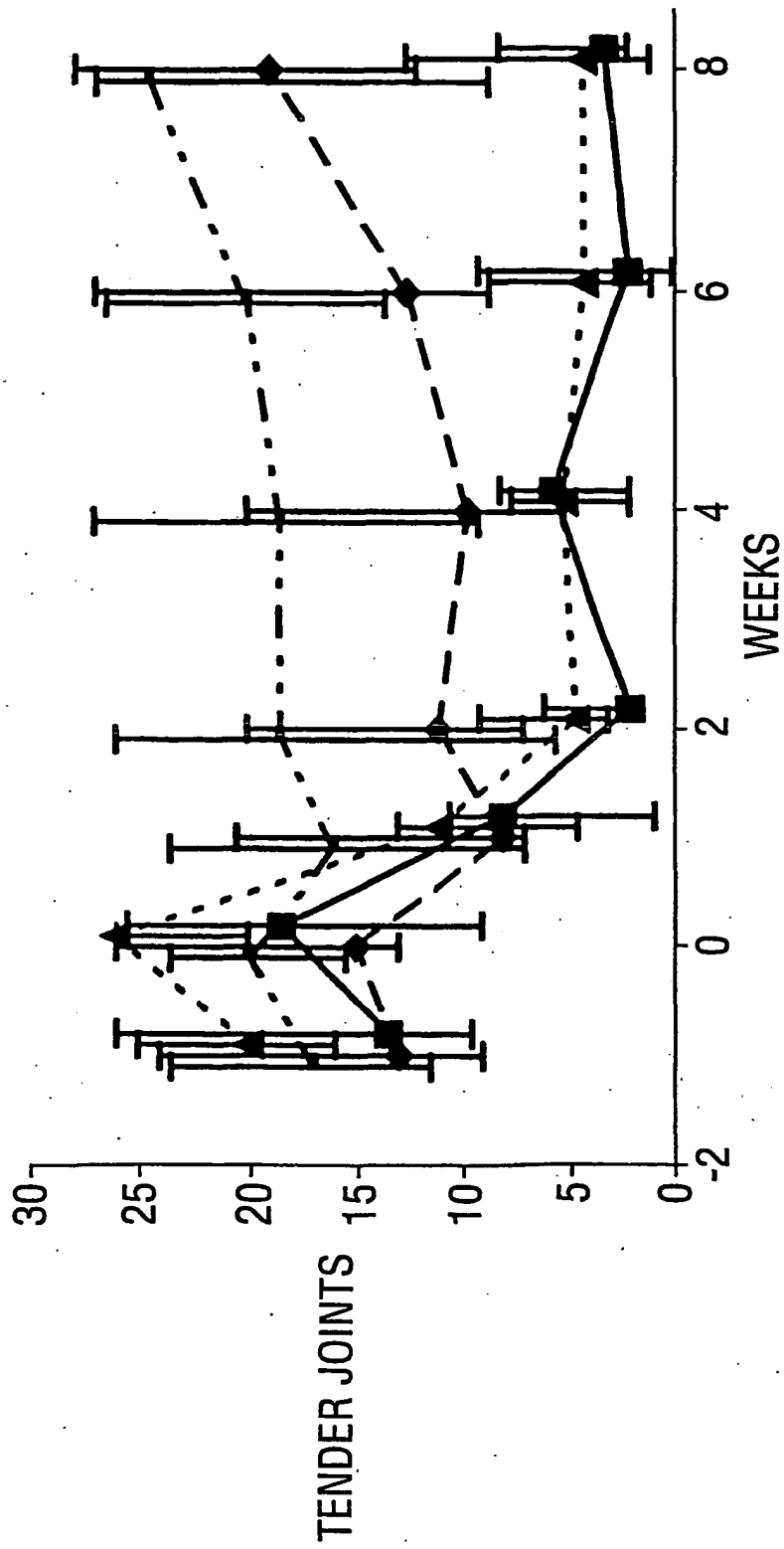
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FIG. 24(contd.)



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FIG. 24(contd.)



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FIG. 24(contd.)

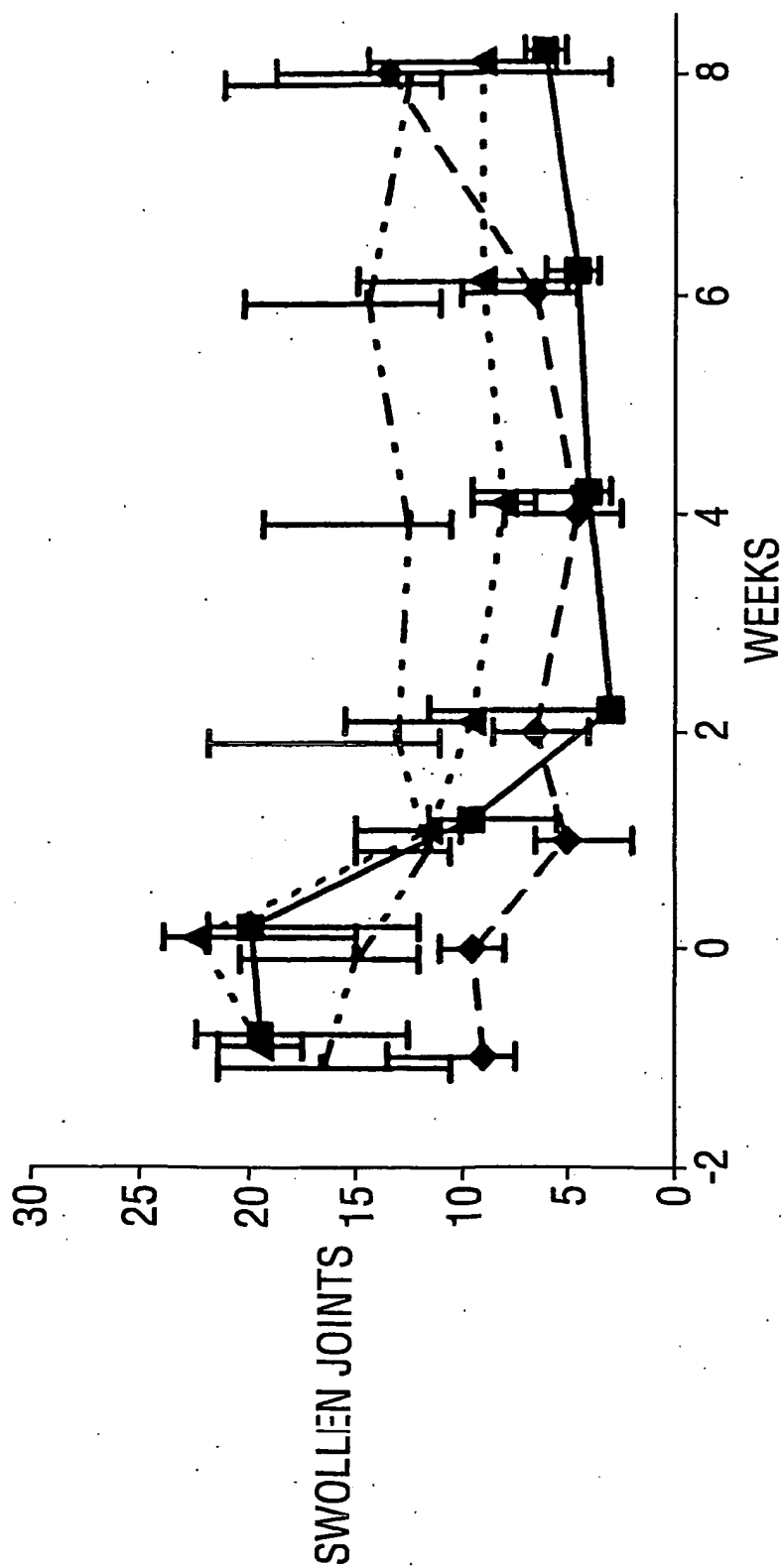


FIG. 24(contd.)

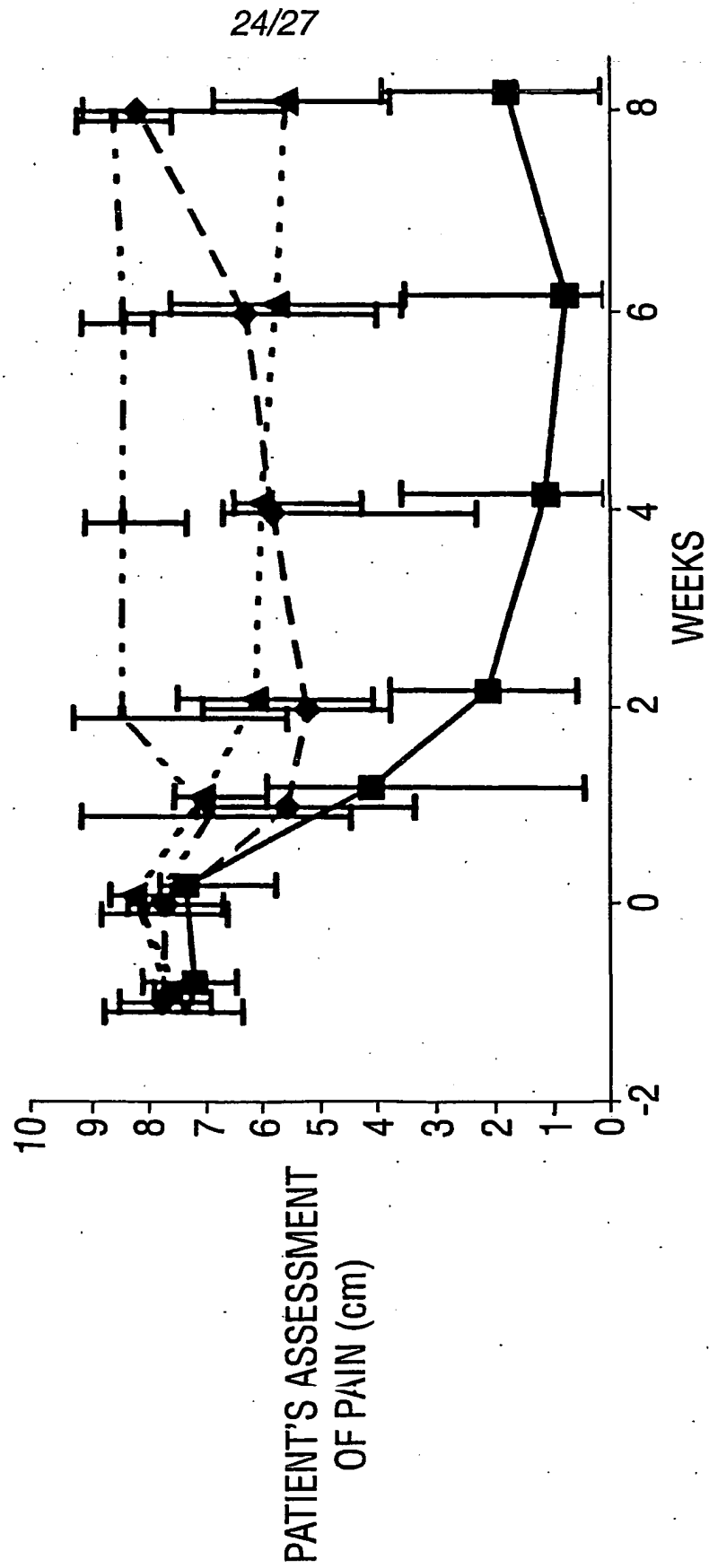
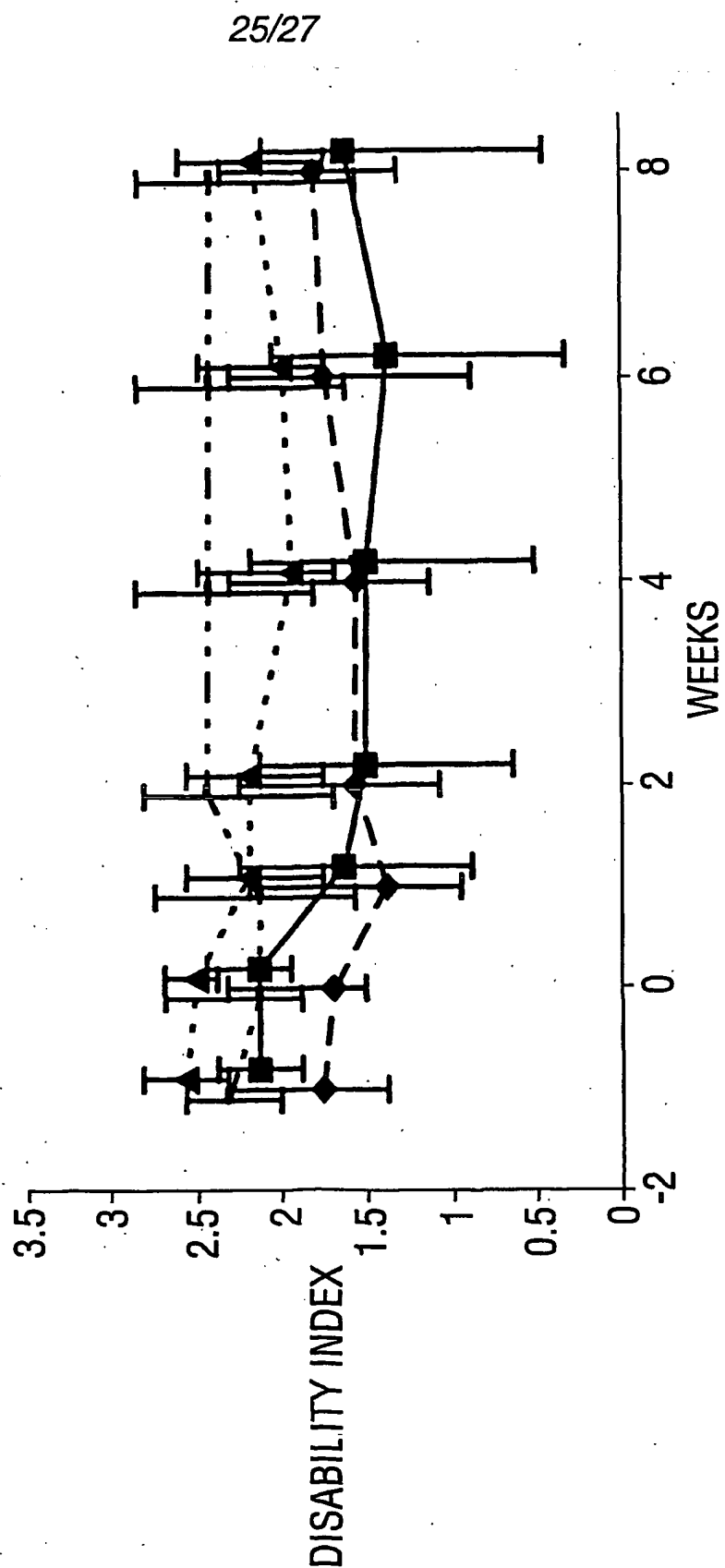
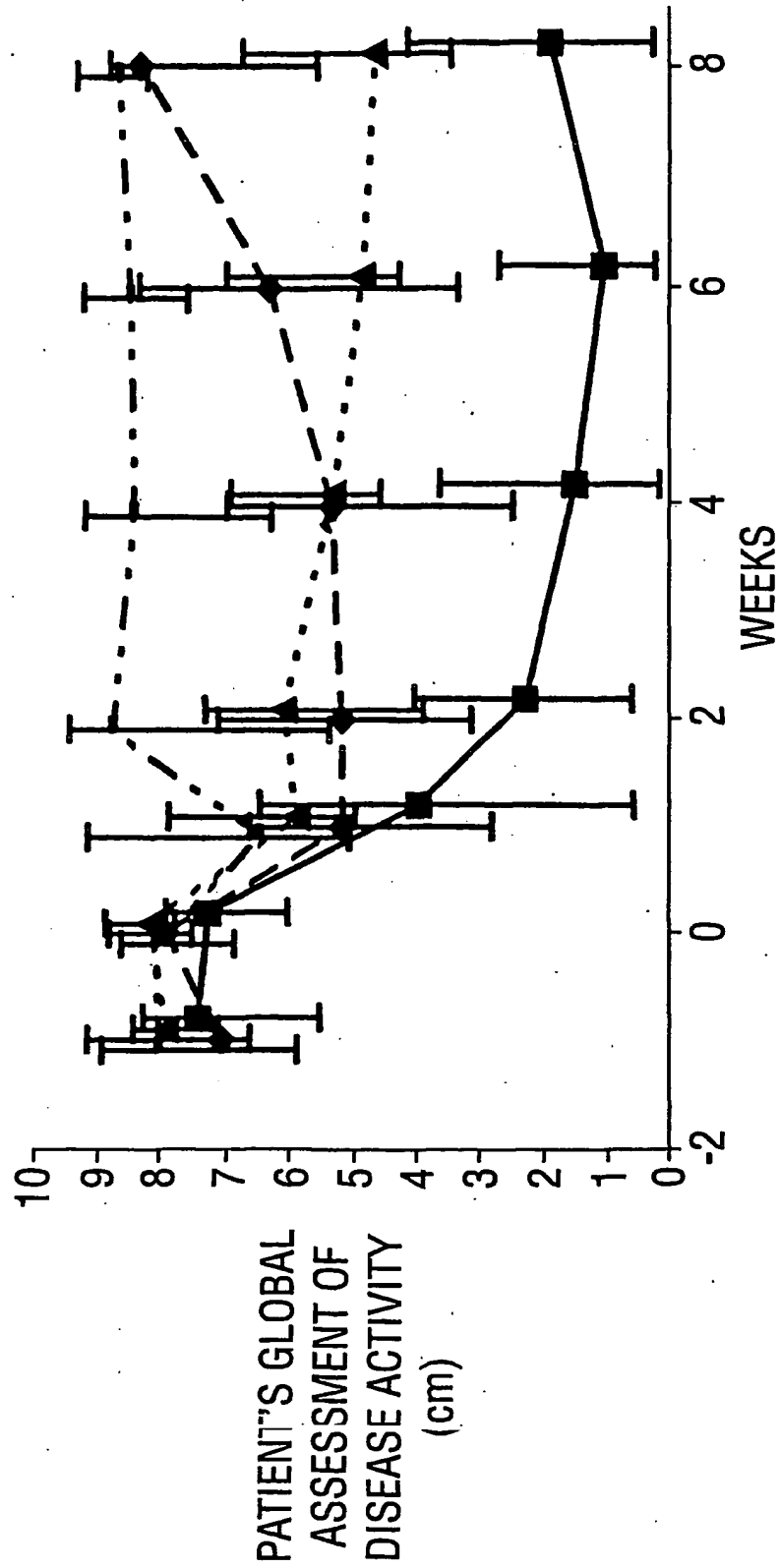


FIG. 24(contd.)



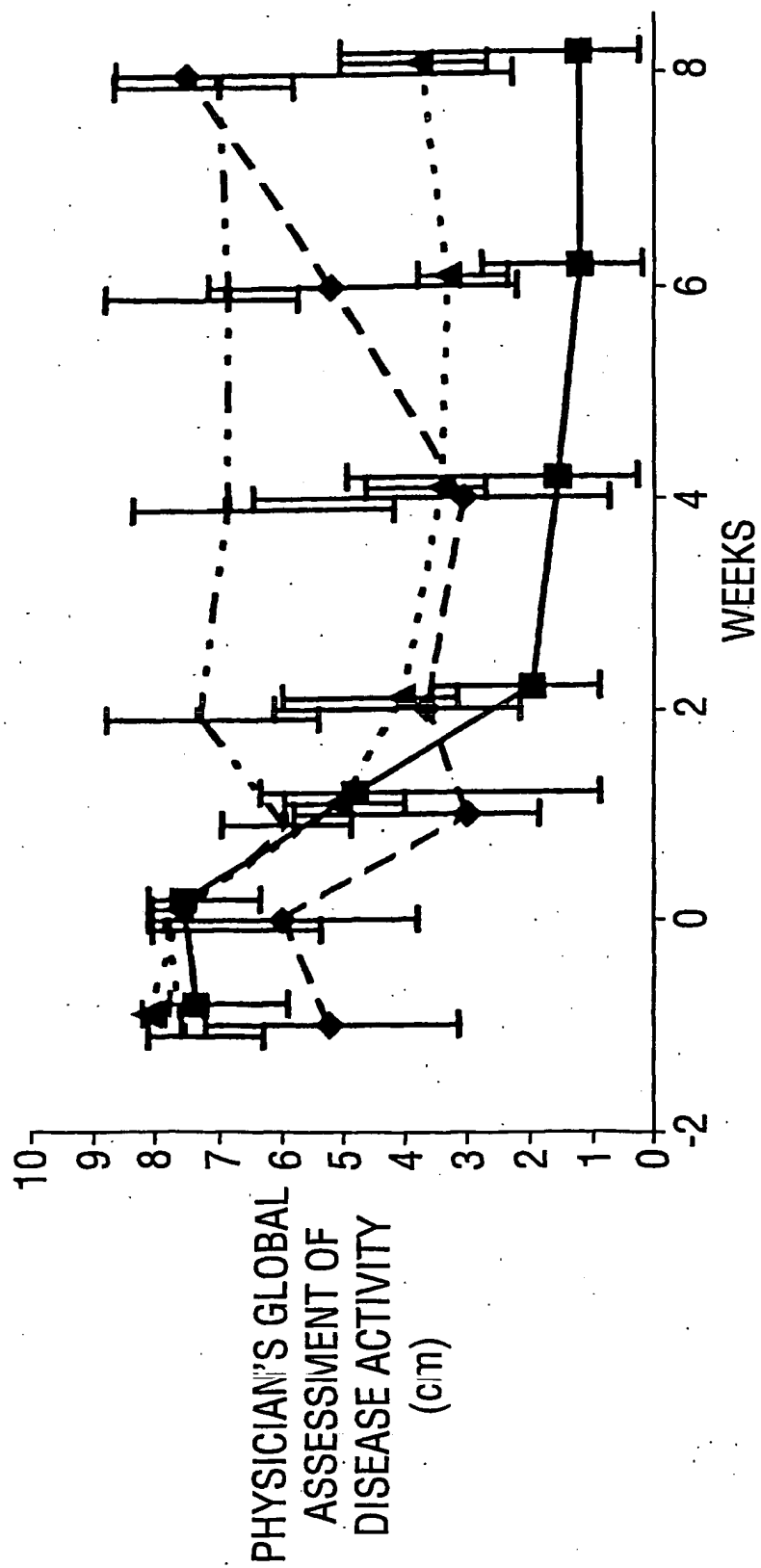
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FIG. 24(contd.)



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FIG. 24(contd.)



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<210> 1

<211> 5

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<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 CDRH1

<400> 1

Asp Tyr Gly Met Asn
1 5

<210> 2

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40/human hybrid
CDRH2

<400> 2

Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val Lys
1 5 10 15

Gly

<210> 3

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 CDRH3

<400> 3

Gly Tyr Arg Ser Tyr Ala Met Asp Tyr
1 5

<210> 4
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRL1

<400> 4
Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala
1 5 10

<210> 5
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRL2

<400> 5
Ser Ala Ser Phe Leu Tyr Ser
1 5

<210> 6
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRL3

<400> 6
Gln Gln Tyr Asn Ile Tyr Pro Leu Thr
1 5

<210> 7
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 CDRH2

<400> 7
Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Val Asp Asp Phe Lys
1 5 10 15

Gly

<210> 8

<211> 321
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(321)

<220>
 <223> Description of Artificial Sequence:hTNF40-gL1

<400> 8
 gac att caa atg acc cag agc cca tcc agc ctg agc gca tct gta gga 48
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 gac cgg gtc acc atc act tgt aaa gcc agt cag aac gta ggt act aac 96
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
 20 25 30
 gta gcc tgg tat cag caa aaa cca ggt aaa gcc cca aaa gcc ctc atc 144
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile
 35 40 45
 tac agt gcc tct ttc ctc tat agt ggt gta cca tac agg ttc agc gga 192
 Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly
 50 55 60
 tcc ggt agt ggt act gat ttc acc ctc acg atc agt agc ctc cag cca 240
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 gaa gat ttc gcc act tat tac tgt caa cag tat aac atc tac cca ctc 288
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu
 85 90 95
 aca ttc ggt cag ggt act aaa gta gaa atc aaa 321
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 9
 <211> 321
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(321)

<220>
 <223> Description of Artificial Sequence:hTNF40-gL2

<400> 9
 gac att caa atg acc cag agc cca tcc agc ctg agc gca tct gta gga 48
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly

1	5	10	15	
gac cgg gtc acc atc act tgt aaa gcc agt cag aac gta ggt act aac	96			
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn				
20 25 30				
gta gcc tgg tat cag caa aaa cca ggt aaa gcc cca aaa ctc ctc atc	144			
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile				
35 40 45				
tac agt gcc tct ttc ctc tat agt ggt gta cca tac agg ttc agc gga	192			
Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly				
50 55 60				
tcc ggt agt ggt act gat ttc acc ctc acg atc agt agc ctc cag cca	240			
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro				
65 70 75 80				
gaa gat ttc gcc act tat tac tgt caa cag tat aac atc tac cca ctc	288			
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu				
85 90 95				
aca ttc ggt cag ggt act aaa gta gaa atc aaa	321			
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys				
100 105				

<210> 10

<211> 354

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(354)

<220>

<223> Description of Artificial Sequence:gh1hTNF40.4 (Figure 10)

<400> 10

cag gtg cag ctg gtc cag tca gga gca gag gtt aag aag cct ggt gct	48
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala	
1 5 10 15	
tcc gtc aaa gtt tcg tgt aag gcc tca ggc tac gtg ttc aca gac tat	96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Val Phe Thr Asp Tyr	
20 25 30	
ggt atg aat tgg gtc aga cag gcc ccg gga caa ggc ctg gaa tgg atg	144
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met	
35 40 45	
ggt tgg att aat act tac att gga gag cct att tat gct caa aag ttc	192
Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Gln Lys Phe	
50 55 60	

```

cag ggc aga gtc acg ttc act cta gac acc tcc aca agc act gca tac 240
Gln Gly Arg Val Thr Phe Thr Leu Asp Thr Ser Thr Ser Thr Ala Tyr
65          70          75          80

atg gag ctg tca tct ctg aga tcc gag gac acc gca gtg tac tat tgt 288
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85          90          95

gct aga gga tac aga tct tat gcc atg gac tac tgg ggc cag ggt acc 336
Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
100        105        110

cta gtc aca gtc tcc tca 354
Leu Val Thr Val Ser Ser
115

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<210> 11
 <211> 354
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(354)

<220>
 <223> Description of Artificial Sequence:gh3hTNF40.4 (Figure 11)

```

<400> 11
gag gtt cag ctg gtc gag tca gga ggc ggt ctc gtg cag cct ggc gga 48
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1          5          10          15

tca ctg aga ttg tcc tgt gct gca tct ggt tac gtc ttc aca gac tat 96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr
20        25        30

gga atg aat tgg gtt aga cag gcc ccg gga aag ggc ctg gaa tgg atg 144
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
35        40        45

ggt tgg att aat act tac att gga gag cct att tat gct gac agc gtc 192
Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val
50        55        60

aag ggc aga ttc acg ttc tct cta gac aca tcc aag tca aca gca tac 240
Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65          70          75          80

ctc caa atg aat agc ctg aga gca gag gac acc gca gtg tac tat tgt 288
Leu Gln Met Asn Ser Leu Arg Ala Gln Asn Thr Ala Val Tyr Tyr Cys
85          90          95

gct aga gga tac aga tct tat gcc atg gac tac tgg ggc cag ggt acc 336

```

Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
100 105 110

cta gtc aca gtc tcc tca
Leu Val Thr Val Ser Ser
115

354

<210> 12
<211> 9
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:part of a primer sequence

<400> 12
gccgccacc

9

<210> 13
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH1

<400> 13
atgaaatgca gctgggtcat sttctt

26

<210> 14
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH2

<400> 14
atgggatgga gctrtatcat sytctt

26

<210> 15
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH3

<400> 15
atgaagwtgt ggtaaactg ggtttt

26

<210> 16
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH4

<400> 16
atgractttg ggytcagctt grt

23

<210> 17
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH5

<400> 17
atggactcca ggctcaattt agtttt

26

<210> 18
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH6

<400> 18
atggctgtcy trsgcttct cttctg

26

<210> 19
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH7

<400> 19
atggratgga gckggrtctt tmtctt

25

<210> 20
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CH8

<400> 20

atgagagtgc tgattctttt gtg

23

<210> 21

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH9

<400> 21

atggmttggg tgtggamctt gctatt

26

<210> 22

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH10

<400> 22

atgggcagac ttacattctc attcct

26

<210> 23

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH11

<400> 23

atggattttg ggctgatttt ttttattg

28

<210> 24

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CH12

<400> 24

atgatggtgt taagtcttct gtacct

26

<210> 25

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer 5' end

<400> 25

gcgcgcaagc ttgccgccac c 21

<210> 26
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL1

<400> 26
atgaagttgc ctgtaggct gttggtgct 29

<210> 27
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL2

<400> 27
atggagwcag acacactcct gytatgggt 29

<210> 28
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL3

<400> 28
atgagtgtgc tcactcaggt cct 23

<210> 29
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL4

<400> 29
atgaggrccc ctgctcagwt tyttgg 26

<210> 30
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL5

<400> 30
atggatttwc aggtgcagat twtcagctt 29

<210> 31
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL5A

<400> 31
atggatttwc argtgcagat twtcagctt 29

<210> 32
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL6

<400> 32
atgaggtkcy ytgytsagyt yctgrg 26

<210> 33
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
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<400> 33
atggggtwca agatggagtc aca 23

<210> 34
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL8

<400> 34
atgtggggay ctktttycmm tttttcaat 29

<210> 35
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
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<400> 35
atggtttccw cagctcagtt cctt 24

<210> 36
<211> 26

<212> DNA
<213> Artificial Sequence

<220>
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<400> 36
atgtatatat gtttgttgtc tatttc 26

<210> 37
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL11

<400> 37
atggaagccc cagctcagct tctctt 26

<210> 38
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL12A

<400> 38
atgragtywc agacccaggt cttyrct 26

<210> 39
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL12B

<400> 39
atggagacac attctcaggt ctttgt 26

<210> 40
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer CL13

<400> 40
atggattcac aggcccaggt tcttat 26

<210> 41
<211> 26
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL14

<400> 41

atgatgagtc ctgcccagtt cctggt

26

<210> 42

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL15

<400> 42

atgaatttgc ctgttcattt cttggtgct

29

<210> 43

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL16

<400> 43

atggattttc aattggtcct catctcctt

29

<210> 44

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL17A

<400> 44

atgagtgcc tarcttsagtt cctgrg

26

<210> 45

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL17B

<400> 45

atgaagtact ctgctcagtt tctagg

26

<210> 46

<211> 26

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer CL17C

<400> 46

atgaggcatt ctcttcaatt cttggg

26

<210> 47

<211> 21

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer 5' end

<400> 47

ggactgttcg aagccgccac c

21

<210> 48

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer CL12

<400> 48

ggatacagtt ggtgcagcat ccgtacgttt

30

<210> 49

<211> 37

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer R2155

<400> 49

gcagatgggc ccttcggtga ggctgmrgag acdgtga

37

<210> 50

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer R1053

<400> 50

gctgacagac taacagactg ttcc

24

<210> 51

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer R720

<400> 51
gctctcggag gtgctcct 18

<210> 52
<211> 70
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7982

<400> 52
gaattcaggg tcaccatcac ttgtaaagcc agtcagaacg taggtactaa cgtagcctgg 60
tattcagcaaa 70

<210> 53
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7983

<400> 53
atagaggaaa gaggcactgt agatgagggc ttttggggct ttacctgggt tttgctgata 60
ccaggctacg t 71

<210> 54
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7984

<400> 54
tacagtgcct ctttcctcta tagtgggtga ccatacaggt tcagcggatc cggtagtggt 60
actgatttca c 71

<210> 55
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligonucleotide P7985

<400> 55
gacagtaata agtggcgaaa tcttctggct ggaggctact gatcgtgagg gtgaaatcag 60
taccactacc g 71

<210> 56
<211> 89
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide P7986

<400> 56

atttcgccac ttattactgt caacagtata acatctaccc actcacattc ggtcagggta 60
ctaaagtaga aatcaaacgt acggaattc 89

<210> 57

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide P7981

<400> 57

gaattcaggg tcaccatcac ttgtaaagcc 30

<210> 58

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide P7980

<400> 58

gaattccgta cgtttgattt ctactttagt 30

<210> 59

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R1053

<400> 59

gctgacagac taacagactg ttcc 24

<210> 60

<211> 57

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R5350

<400> 60

tctagatggc acaccatctg ctaagtttga tgcagcatag atcaggagct taggagc 57

<210> 61

<211> 59

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R5349

<400> 61

gcagatgggtg tgccatctag attcagtggc agtggatcag gcacagactt taccctaac 59

<210> 62

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide R684

<400> 62

ttcaactgct catcagat

18

<210> 63

<211> 65

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7989

<400> 63

gaagcaccag gcttcttaac ctctgctcct gactggacca gctgcacctg agagtgcacg 60
aatc 65

<210> 64

<211> 71

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7990

<400> 64

ggttaagaag cctggtgctt ccgtcaaagt ttctgtgaag gcctcaggct acgtgttcac 60
agactatggt a 71

<210> 65

<211> 71

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7991

<400> 65

ccaaccatc catttcaggc cttgtcccgg ggctgcttg acccaattca taccatagtc 60
tgtgaacacg t 71

<210> 66

<211> 81

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7995

<400> 66

ggcctgaaat ggatgggttg gattaatact tacattggag agcctattta tgttgacgac 60
ttcaagggca gattcacgtt c 81

<210> 67

<211> 56

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7992

<400> 67

ccatgtatgc agtgcgttgt ggaggtgtct agagtgaacg tgaatctgcc cttgaa 56

<210> 68

<211> 62

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7993

<400> 68

ccacaagcac tgcatacatg gagctgtcat ctctgagatc cgaggacacc gcagtgtact 60
at 62

<210> 69

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7994

<400> 69

gaattcggtg ccctggcccc agtagtccat ggcataagat ctgtatcctc tagcacaata 60
gtacactgcg gtgtcctc 78

<210> 70

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7988

<400> 70

gaattcgtgc actctcaggt gcagctggtc 30

<210> 71

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7987

<400> 71

gaattcggta ccctggcccc agtagtccat

30

<210> 72

<211> 65

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer P7999

<400> 72

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aattc 65

<210> 73

<211> 71

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer P8000

<400> 73

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agactatgga a 71

<210> 74

<211> 71

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer P8001

<400> 74

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tgtgaagacg t 71

<210> 75

<211> 55

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer P7997

<400> 75

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<210> 76

<211> 62

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer P7998

<400> 76
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at 62

<210> 77
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer P7993

<400> 77
gaattcggtg ccctggcccc agtagtccat ggcataagat ctgtatcctc tagcacaata 60
gtacactgcg gtgtcctc 78

<210> 78
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer P7996

<400> 78
gaattcgtgc actctgaggt tcagctggtc 30

<210> 79
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 5' primer

<400> 79
cgcgcggaac ttgcagtggc cttggctggt ttcgctaccg tagcgcaagc tgacattcaa 60
atgacccaga gcc 74

<210> 80
<211> 20
<212> DNA
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<220>
<223> Description of Artificial Sequence: 3' primer

<400> 80
ttcaactgct catcagatgg 20

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 <211> 78
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: 5' primer

<400> 81
 gctatcgcaa ttgcagtggc gctagctggt ttcgccaccg tggcgcaagc tgaggttcag 60
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<210> 82
 <211> 18
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: 3' primer

<400> 82
 gcctgagttc cacgacac 18

<210> 83
 <211> 23
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: human group 1 consensus
 framework L1

<400> 83
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15

Asp Arg Val Thr Ile Thr Cys
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<210> 84
 <211> 23
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: hTNF40 framework L1

<400> 84
 Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15

Asp Arg Val Ser Val Thr Cys
 20

<210> 85
 <211> 15
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 1 consensus
 framework L2

<400> 85
 Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
 1 5 10 15

<210> 86
 <211> 15
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:hTNF40 framework L2

<400> 86
 Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile Tyr
 1 5 10 15

<210> 87
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 1 consensus
 framework L3

<400> 87
 Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
 1 5 10 15

Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
 20 25 30

<210> 88
 <211> 32
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:hTNF40 framework L3

<400> 88
 Gly Val Pro Tyr Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr
 1 5 10 15

Leu Thr Ile Ser Thr Val Gln Ser Glu Asp Leu Ala Glu Tyr Phe Cys
 20 25 30

<210> 89

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus
 framework L4

<400> 89

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
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<210> 90

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework L4

<400> 90

Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
 1 5 10

<210> 91

<211> 30

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus
 framework H1

<400> 91

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
 20 25 30

<210> 92

<211> 30

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework H1

<400> 92

Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
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Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Val Phe Thr
20 25 30

<210> 93

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus
framework H2

<400> 93

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
1 5 10

<210> 94

<211> 14

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:hTNF40 framework H2

<400> 94

Trp Val Lys Gln Ala Pro Gly Lys Ala Phe Lys Trp Met Gly
1 5 10

<210> 95

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 1 consensus
framework H3

<400> 95

Arg Val Thr Ile Thr Arg Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
1 5 10 15

Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 96

<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 framework H3

<400> 96
Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Phe Leu Gln
1 5 10 15
Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr Tyr Phe Cys Ala Arg
20 25 30

<210> 97
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:human group 1 consensus
framework H4

<400> 97
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
1 5 10

<210> 98
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:hTNF40 framework H4

<400> 98
Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser
1 5 10

<210> 99
<211> 324
<212> DNA
<213> murine

<220>
<221> CDS
<222> (1) (324)
<223> mouse hTNF40 light chain variable domain

<400> 99

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 Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15
 gac agg gtc agc gtc acc tgc aag gcc agt cag aat gtg ggt act aat 96
 Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
 20 25 30
 gta gcc tgg tat caa cag aaa cca gga caa tct cct aaa gca ctg att 144
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile
 35 40 45
 tac tcg gca tcc ttc cta tat agt gga gtc cct tat cgc ttc aca ggc 192
 Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Thr Gly
 50 55 60
 agt gga tct ggg aca gat ttc act ctc acc atc agc act gtg cag tct 240
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Thr Val Gln Ser
 65 70 75 80
 gaa gac ttg gca gag tat ttc tgt cag caa tat aac atc tat cct ctc 288
 Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ile Tyr Pro Leu
 85 90 95
 acg ttc ggt gct ggg acc aag ctg gag ctg aaa cgt 324
 Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
 100 105

<210> 100
 <211> 354
 <212> DNA
 <213> murine

<220>
 <221> CDS
 <222> (1)..(354)
 <223> mouse hTNF40 heavy chain variable domain

<400> 100
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 Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu
 1 5 10 15
 aca gtc aag atc tcc tgc aag gct tct gga tat gtt ttc aca gac tat 96
 Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Val Phe Thr Asp Tyr
 20 25 30
 gga atg aat tgg gtg aag cag gct cca gga aag gct ttc aag tgg atg 144
 Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Ala Phe Lys Trp Met
 35 40 45
 gcc tgg ata aac acc tac att gga gag cca ata tat gtt gat gac ttc 192
 Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Val Asp Asp Phe
 50 55 60

aag gga cga ttt gcc ttc tct ttg gaa acc tct gcc agc act gcc ttt 240
 Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Phe
 65 70 75 80

ttg cag atc aac aac ctc aaa aat gag gac acg gct aca tat ttc tgt 288
 Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr Tyr Phe Cys
 85 90 95

gca aga ggt tac cgg tcc tat gct atg gac tac tgg ggt caa gga acc 336
 Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
 100 105 110

tca gtc acc gtc tct tca 354
 Ser Val Thr Val Ser Ser
 115

<210> 101
 <211> 84
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (29)..(67)

<223> Description of Artificial Sequence:OmpA oligonucleotide adaptor

<400> 101
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 Met Lys Lys Thr Ala Ile Ala Ile
 1 5

gca gtg gcc ttg gct ctgacgtacg agtcagg 84
 Ala Val Ala Leu Ala
 10

<210> 102
 <211> 67
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (2)..(40)

<220>
 <221> CDS
 <222> (43)..(66)

<220>
 <223> Description of Artificial Sequence:IGS cassette-1

<400> 102
 g agc tca cca gta aca aaa agt ttt aat aga gga gag tgt ta atg aag 48

Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Xaa Xaa Lys
 1 5 10 15
 aag act gct ata gca att g 67
 Lys Thr Ala Ile Ala Ile
 20

<210> 103
 <211> 69
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (2)..(43)

<220>
 <221> CDS
 <222> (45)..(68)

<220>
 <223> Description of Artificial Sequence:IGS cassette-2

<400> 103
 g agc tca cca gta aca aaa agt ttt aat aga ggg gag tgt taa a atg 47
 Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys Met
 1 5 10 15
 aag aag act gct ata gca att g 69
 Lys Lys Thr Ala Ile Ala Ile
 20

<210> 104
 <211> 81
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (2)..(43)

<220>
 <221> CDS
 <222> (57)..(80)

<220>
 <223> Description of Artificial Sequence:IGS cassette-3

<400> 104
 g agc tca cca gta aca aaa agc ttt aat aga gga gag tgt tga 43
 Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
 1 5 10
 ggaggaaaaaaa aaa atg aag aaa act gct ata gca att g 81
 Met Lys Lys Thr Ala Ile Ala Ile

28

15

20

<210> 105
 <211> 81
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (2)..(43)

<220>
 <221> CDS
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<220>
 <223> Description of Artificial Sequence:IGS cassette-4

<400> 105
 g agc tca cca gta aca aaa agt ttt aat aga gga gag tgt tga 43
 Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
 1 5 10

cgaggattat ata atg aag aaa act gct ata gca att g 81
 Met Lys Lys Thr Ala Ile Ala Ile
 15 20

<210> 106
 <211> 30
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 3 consensus
 framework H1

<400> 106
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
 20 25 30

<210> 107
 <211> 13
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:human group 3 consensus
 framework H2

<400> 107

Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ser
 1 5 10

<210> 108

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 3 consensus framework H3

<400> 108

Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln
 1 5 10 15

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
 20 25 30

<210> 109

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:human group 3 consensus framework H4

<400> 109

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
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<210> 110

<211> 648

<212> DNA

<213> Artificial Sequence

<220>

<223> Grafted Heavy Chain for Fab

<400> 110

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ccgggaaagg	gcctggaatg	gatgggttgg	attaataact	acattggaga	gcctatztat	180
gctgacagcg	tcaagggcag	attcacgttc	tctctagaca	catccaagtc	aacagcatac	240
ctccaaatga	atagcctgag	agcagaggac	accgcagtgt	actattgtgc	tagaggatac	300
agatcttatg	ccatggacta	ctggggccag	ggtaccctag	tcacagtctc	ctcagcttcc	360
accaaggggc	catcggtctt	ccccctggca	ccctcctcca	aaagcacctc	tgggggcaca	420
gcggccctgg	gctgcctggc	caaggactac	ttccccgaac	cggtgacggc	gtcgtggaac	480
tcaggcgccc	tgaccagcgg	cgtgcacacc	ttcccggctg	tcctacagtc	ctcaggactc	540
tactccctca	gcagcgtggc	gaccgtgccc	tccagcagct	tgggcaccca	gacctacatc	600

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648

<210> 111
 <211> 216
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Grafted Heavy Chain for Fab

<400> 111
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
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 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr
 20 25 30
 Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
 35 40 45
 Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
 100 105 110
 Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro
 115 120 125
 Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
 130 135 140
 Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn
 145 150 155 160
 Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln
 165 170 175
 Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser
 180 185 190
 Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
 195 200 205
 Asn Thr Lys Val Asp Lys Lys Val
 210 215

<210> 112
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<223> Grafted Light Chain for Fab and Modified Fab

<400> 112

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ggtaaagccc caaaagccct catctacagt gcctctttcc tctatagtgg tgtaccatac 180
aggttcagcg gatccggtag tggtagtgat ttcaccctca cgatcagtag cctccagcca 240
gaagatttcg ccacttatta ctgtcaacag tataacatct acccactcac attcggtcag 300
ggtactaaag tagaaatcaa acgtacggta gcggcccat ctgtcttcat ctccccgcca 360
tctgatgagc agttgaaatc tggaactgcc tctgttgtgt gcctgctgaa taacttctat 420
cccagagagg ccaaagtaca gtggaagggtg gataacgccc tccaatcggg taactcccag 480
gagagtgtca cagagcagga cagcaaggac agcacctaca gcctcagcag caccctgacg 540
ctgagcaaag cagactacga gaaacacaaa gtctacgcct gcgaagtcac ccatcagggc 600
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<210> 113

<211> 214

<212> PRT

<213> Artificial Sequence

<220>

<223> Grafted Light Chain for Fab and Modified Fab

<400> 113

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Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
          20           25           30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Ala Leu Ile
          35           40           45

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Tyr Arg Phe Ser Gly
          50           55           60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65           70           75           80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ile Tyr Pro Leu
          85           90           95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
          100          105          110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
          115          120          125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
          130          135          140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145          150          155          160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser

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165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
195 200 205

Phe Asn Arg Gly Glu Cys
210

<210> 114
<211> 687
<212> DNA
<213> Artificial Sequence

<220>
<223> Grafted Heavy Chain for Modified Fab

<400> 114

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tcctgtgctg	catctgggta	cgtcttcaca	gactatggaa	tgaattgggt	tagacaggcc	120
ccgggaaagg	gcctggaatg	gatgggttgg	attaataactt	acattggaga	gcctatztat	180
gctgacagcg	tcaagggcag	attcacgttc	tctctagaca	catccaagtc	aacagcatatc	240
ctccaaatga	atagcctgag	agcagaggac	accgcagtgt	actattgtgc	tagaggatac	300
agatcttatg	ccatggacta	ctggggccag	ggtaccctag	tcacagtctc	ctcagcttcc	360
accaagggcc	catcggtctt	ccccctggca	ccctcctcca	agagcacctc	tgggggcaca	420
gcggccctgg	gctgcctgg	caaggactac	ttccccgaac	cggtgacgg	gtcgtggaac	480
tcaggcgccc	tgaccagcgg	cgtgcacacc	ttcccggtg	tcctacagtc	ctcaggactc	540
tactccctca	gcagcgtgg	gaccgtgccc	tccagcagct	tgggcaccca	gacctacatc	600
tgaacgtga	atcacaagcc	cagcaacacc	aaggtcgaca	agaaagttga	gcccaaatct	660
tgtgacaaaa	ctcacacatg	cgccgcg				687

<210> 115
<211> 229
<212> PRT
<213> Artificial Sequence

<220>
<223> Grafted Heavy Chain for Modified Fab

<400> 115

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1 5 10 15	
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Val Phe Thr Asp Tyr	
20 25 30	
Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met	
35 40 45	
Gly Trp Ile Asn Thr Tyr Ile Gly Glu Pro Ile Tyr Ala Asp Ser Val	
50 55 60	
Lys Gly Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr	
65 70 75 80	

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Tyr Arg Ser Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr
 100 105 110
 Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro
 115 120 125
 Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
 130 135 140
 Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn
 145 150 155 160
 Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln
 165 170 175
 Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser
 180 185 190
 Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
 195 200 205
 Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
 210 215 220
 His Thr Cys Ala Ala
 225

INTERNATIONAL SEARCH REPORT

In International Application No

PCT/GB 01/02477

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/13 C07K16/24 C07K16/46 A61K47/48 C07K19/00
 C12N15/62 C12N15/70 C12N1/21 A61K39/395 A61P19/02
 A61P37/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 25971 A (CELLTECH THERAPEUTICS LIMITED) 18 June 1998 (1998-06-18)	1-4, 13, 15, 16, 26, 28-32, 60-64, 67
Y	examples 2, 5, 6 claims	6-12, 14, 17-23, 27, 33-41, 49-54, 56-59

	-/-	



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Patent family members are listed in annex.

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Date of the actual completion of the international search

15 August 2001

Date of mailing of the international search report

22/08/2001

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 01/02477

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 92 11383 A (CELLTECH LIMITED) 9 July 1992 (1992-07-09)</p> <p>examples claims</p>	<p>6-12,14, 17-23, 27, 33-41, 49-54, 56-59</p>
X	<p>WO 99 64460 A (CELLTECH THERAPEUTICS LIMITED) 16 December 1990 (1990-12-16)</p> <p>examples 3,4 claims</p>	<p>1-4,13, 15,16, 26, 28-32, 60-64,67</p>
X	<p>EP 0 380 068 A (MOLECULAR THERAPEUTICS, INC.) 1 August 1990 (1990-08-01) figure 4</p>	<p>67</p>
A	<p>S. STEPHENS ET AL.: "Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses." IMMUNOLOGY, vol. 85, no. 4, August 1995 (1995-08), pages 668-674, XP000881488 Oxford, GB abstract page 668, right-hand column figure 1</p>	<p>1-67</p>
A	<p>K. NAGAHIRA ET AL.: "Humanization of a mouse neutralizing monoclonal antibody against tumor necrosis factor-alpha (TNF-alpha)." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 222, no. 1-2, 1 January 1999 (1999-01-01), pages 83-92, XP004152430 Amsterdam, The Netherlands abstract figure 2</p>	<p>1-67</p>

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PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 7/06, 7/08, 7/10 C07K 7/64, A61K 37/02	A1	(11) International Publication Number: WO 93/06128 (43) International Publication Date: 1 April 1993 (01.04.93)
(21) International Application Number: PCT/AU92/00487 (22) International Filing Date: 16 September 1992 (16.09.92) (30) Priority data: PK 8367 16 September 1991 (16.09.91) AU (71) Applicant (for all designated States except US): PEPTIDE TECHNOLOGY LIMITED [AU/AU]; 4-10 Inman Road, Dee Why, NSW 2099 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only) : RATHJEN, Deborah, Ann [AU/AU]; 4 Eddy Street, Thornleigh, NSW 2120 (AU). WIDMER, Fred [CH/AU]; 35 Anzac Avenue, Ryde, NSW 2112 (AU). (74) Agent: F B RICE & CO.; P.O. Box 117, 28a Montague Street, Balmain, NSW 2041 (AU).		(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: TNF ANTAGONIST PEPTIDES (57) Abstract The present invention provides TNF antagonist peptides which have the ability to reduce TNF toxicity. The present invention further relates to compositions including these peptides as the active ingredient and to methods of treatment involving the administration of this composition.		

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TNF ANTAGONIST PEPTIDESField of the Invention

The present invention relates to TNF antagonist peptides which have the ability to reduce TNF toxicity as manifest by decreased blood glucose levels and weight loss (cachexia). The present invention further relates to compositions including these peptides as the active ingredient and methods of treatment involving the administration of this composition.

10 Background of the invention

Many of the clinical features of Gram-negative septicemic shock may be reproduced in animals by the administration of bacterial lipopolysaccharide (LPS). The administration of LPS to animals can prompt severe metabolic and physiological changes which can lead to death. Associated with the injection of LPS is the extensive production of tumour necrosis factor alpha (TNF). Mice injected with recombinant human TNF develop piloerection of the hair (ruffling), diarrhea, a withdrawn, unkempt appearance and die if sufficient amounts are given. Rats treated with TNF become hypotensive, tachypneic and die of sudden respiratory arrest (Tracey et al., 1986 Science 234, 470). Severe acidosis, marked hemoconcentration and biphasic changes in blood glucose concentration were also observed. Histopathology revealed severe leukostasis in the lungs, haemorrhagic necrosis in the adrenals, pancreas and other organs and tubular necrosis of the kidneys. All these changes were prevented if the animals were pretreated with a neutralizing monoclonal antibody against TNF.

The massive accumulation of neutrophils in the lungs of TNF-treated animals reflects the activation of neutrophils by TNF. TNF causes neutrophil degranulation, respiratory burst, enhanced antimicrobicidal and anti-tumour activity (Klebanoff et al., 1986 J. Immunol.

136, 4220; Tsujimoto et al., 1986 Biochem Biophys Res Commun 137, 1094). Endothelial cells are also an important target for the expression of TNF toxicity. TNF diminishes the anticoagulant potential of the endothelium, inducing procoagulant activity and down regulating the expression of thrombomodulin (Stern and Nawroth, 1986 J Exp Med 163, 740).

TNF, a product of activated macrophages produced in response to infection and malignancy, was first identified as a serum factor in LPS treated mice which caused the haemorrhagic necrosis of transplantable tumours in murine models and was cytotoxic for tumour cells in culture (Carswell et al., 1975 PNAS 72, 3666; Helson et al., 1975 Nature 258, 731). Cachexia is a common symptom of advanced malignancy and severe infection. It is characterised by abnormal lipid metabolism with hypertriglyceridemia, abnormal protein and glucose metabolism and body wasting. Chronic administration of TNF (also known as cachectin in the early literature) to mice causes anorexia, weight loss and depletion of body lipid and protein within 7 to 10 days (Cerami et al., 1985 Immunol Lett 11, 173, Fong et al., 1989 J Exp Med 170, 1627). These effects were reduced by concurrent administration of antibodies against TNF, TNF has been measured in the serum of patients with cancer and chronic disease associated with cachexia. The results are inconclusive since large differences in TNF levels have been reported. These may be due to the short half-life of TNF (6 minutes), differences in TNF serum binding protein or true differences in TNF levels in chronic disease states.

TNF α , as a mediator of inflammation, has been implicated in the pathology of other diseases apart from toxic shock and cancer related cachexia. TNF has been measured in synovial fluid in patients with both

rheumatoid and reactive arthritis and in the serum of patients with rheumatoid arthritis (Saxne et al., 1988 Arthrit. Rheumat. 31, 1041). Raised levels of TNF have been detected in renal transplant patients during acute rejection episodes (Maury and Teppo 1987 J. Exp Med 166, 1132). In animals TNF has been shown to be involved in the pathogenesis of graft versus host disease in skin and gut following allogeneic marrow transplantation. Administration of a rabbit anti-murine TNF was demonstrated to prevent the histological changes associated with graft versus host disease and reduced mortality (Piquet et al., 1987 J Exp Med 166, 1280).

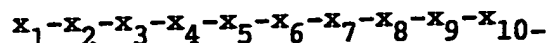
TNF has also been shown to contribute significantly to the pathology of malaria (Clark et al., 1987; Am. J. Pathol. 129: 192-199). Further, elevated serum levels of TNF have been reported in malaria patients (Scuderi et al., 1986; Lancet 2: 1364-1365). TNF may also contribute to the brain pathology and consequent dementia observed in late stage HIV infections (Grimaldi et al Ann Nevrol 29 : 21)

The biological response to TNF is mediated by specific cell surface receptors. At least two cell surface molecules of molecular weight 55 and 75 kd specifically bind TNF α and TNF β with high affinity (Hohmann et al, 1989 J. biol. Chem. 264 14927). These receptors have now been cloned (Loetscher et al, 1990 Cell 61, 351; Smith et al, 1990 Science 248 1019). Both TNF type I and II receptors are shed into serum following proteolytic cleavage to form serum binding proteins which reversibly inactivate TNF and which may regulate the activity of TNF in vivo (Kohn et al., 1990 PNAS 87 8331; Seckinger et al, 1988 J Biol Chem 264 11966). Infusion of TNF has been shown to cause an increase in circulating TNF-binding protein in humans (Lantz et al, 1990 Cytokine 2 1).

The present inventors have produced peptides which are able to reduce TNF toxicity as manifest by reduced mortality decreased blood glucose levels and weight loss (cachexia) in tumour-bearing mice treated with human recombinant TNF.

Summary of the Invention

In a first aspect the present invention consists in a linear or cyclic peptide of the general formula:-



10 in which x_1 is null, Cys or R_1 ;

x_2 is null, Cys, R_1 or

$A_1-A_2-A_3-A_4-A_5-A_6-A_7$

in which A_1 is Ser or Thr or Ala,

A_2 is Lys or Arg or His,

15 A_3 is Cys or Arg or His,

A_4 is His or Lys or Arg or Phe or Tyr or Trp,

A_5 is Lys or Arg or His,

A_6 is Gly or Ala,

A_7 is Thr or Ser or Ala,

20 x_3 is Null, Cys, R_1 or A_8-A_9

in which A_8 is absent or Gly or Ala or Tyr or Phe or Trp or His

A_9 is Leu or Ile or Val or Met

x_4 is Cys, R_1 or

25 $A_{10}-A_{11}-A_{12}-A_{13}-A_{14}$ in which A_{10} is

Tyr or Phe or Trp or His or Gly or Ala,

A_{11} is Asn or Gln,

A_{12} is Asp or Glu,

A_{13} is Cys or Arg or His,

30 A_{14} is Pro or $N\alpha$ - alkyl amino acid

x_5 is Gly or Ala,

x_6 is Cys, R_2 or $A_{15}-A_{16}$

in which A_{15} is Pro or $N\alpha$ - alkyl amino acid, A_{16} is Gly or Ala,

35 x_7 is null, Cys, R_2 or $A_{17}-A_{18}-A_{19}$

- 5 -

in which A₁₇ is Gln or Asn,
 A₁₈ is Asp or Glu,
 A₁₉ is Thr or Ser or Ala,
 X₈ is null, Cys, R₂, Asp, Glu, Gly or Ala,
 5 X₉ is null, Cys, R₂ or
 A₂₀-A₂₁-A₂₂-A₂₃-A₂₄-A₂₅-A₂₆-A₂₇
 -A₂₈-A₂₉

in which A₂₀ is Cys or Arg or His,
 A₂₁ is Arg or Lys or His,
 10 A₂₂ is Glu or Asp,
 A₂₃ is Cys or Arg or His,
 A₂₄ is Glu or Asp,
 A₂₅ is Ser or Thr or Ala,
 A₂₆ is Gly or Ala,
 15 A₂₇ is Ser or Thr or Ala,
 A₂₈ is Phe or Tyr or Trp or His,
 A₂₉ is Thr or Ser or Ala,
 X₁₀ is null, Cys or R₂

R₁ is R-CO, where R is H, straight, branched or cyclic
 20 alkyl up to C20, optionally containing double bonds and/or
 substituted with halogen, nitro, amino, hydroxy, sulfo,
 phospho or carboxyl groups (which may be substituted
 themselves), or aralkyl or aryl optionally substituted as
 listed for the alkyl and further including alkyl, or R₁
 25 is glycosyl, nucleosyl, lipoyl or R₁ is an L- or D- α
 amino acid or an oligomer thereof consisting of up to 5
 residues R₁ is absent when the amino acid adjacent is a
 desamino-derivative.

R₂ is -NR₁₂R₁₃, wherein R₁₂ and R₁₃ are
 30 independently H, straight, branched or cyclic alkyl,
 aralkyl or aryl optionally substituted as defined for R₁
 or N-glycosyl or N-lipoyl -OR₁₄, where R₁₄ is H,
 straight, branched or cyclic alkyl, aralkyl or aryl,
 optionally substituted as defined for R₁-O-glycosyl,
 35 -O-lipoyl or - an L- or D- α -amino acid or an oligomer

thereof consisting of up to 5 residues or R_2 is absent, when the adjacent amino acid is a decarboxy derivative of cysteine or a homologue thereof or the peptide is in a N-C cyclic form.

5 with the proviso that:

X_1 is always and only null when X_2 is R_1 , Cys or null

X_2 is always and only null when X_3 is R_1 , Cys or null

X_3 is always and only null when X_4 is R_1 or Cys

X_7 is always and only null when X_6 is R_2 or Cys

10 X_8 is always and only null when X_7 is null, R_2 or Cys

X_9 is always and only null when X_8 is null, R_2 or Cys

X_{10} is always and only null when X_9 is null, R_2 or Cys

when X_4 is R_1 or Cys then X_6 is A_{15} - A_{16} , X_7 is

15 A_{17} - A_{18} - A_{19} and X_8 is Asp, Glu, Gly or Ala,

when X_6 is R_2 or Cys then X_4 is

A_{10} - A_{11} - A_{12} - A_{13} - A_{14}

when X_7 is null, R_2 or Cys then X_4 is

A_{10} - A_{11} - A_{12} - A_{13} - A_{14} .

20 In a preferred embodiment of the first aspect of the present invention the peptide is selected from the group consisting of:-

H-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp-OH,

25 Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gln-Asp-Thr-Asp-Cys(Acm)-Arg-Glu-Cys(Acm)-Glu-Ser-Gly-Ser-Phe-Thr, Thr-Lys-Cys(Acm)-His-Lys-Gly-Thr-Tyr-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp, Ac-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly,

30 Gly-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly,

Gly-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly,

Gly-Pro-Gly-Gln-Asp-Thr-Asp,

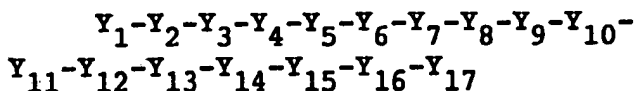
Ac-Gly-Pro-Gly-Gln-Asp-Thr-Asp-NH₂,

Gly-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Gly, and

35 Ac-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-NH₂

In a particularly preferred embodiment of the present invention the peptide is H-Leu-Tyr-Asn-Asp-Cys-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp-OH.

In a second aspect the present invention consists in
5 a linear or cyclic peptide of the general formula:-



in which Y_1 is null, Cys or R_1

Y_2 is Gly or Ala

10 Y_3 is Ala or Glu

Y_4 is Gln or Asn

Y_5 is Met or Gal or Ile or Leu

Y_6 is Cys or Arg or His

Y_7 is Cys or Arg or His

15 Y_8 is Ser or Thr or Ala

Y_9 is Lys or Arg or His

Y_{10} is Cys or Arg or His

Y_{11} is Ser or Thr or Ala

Y_{12} is Pro or α - alkyl amino acid

20 Y_{13} is Gly or Ala

Y_{14} is Gln or Asn

Y_{15} is His or Lys or Arg

Y_{16} is Gly or Ala

Y_{17} is null, Cys or A_2

25 R_1 is R-CO, where R is H, straight, branched or cyclic alkyl up to C20, optionally containing double bonds and/or substituted with halogen, nitro, amino, hydroxy, sulfo, phospho or carboxyl groups (which may be substituted themselves), or aralkyl or aryl optionally substituted as
30 listed for the alkyl and further including alkyl, or R_1 is glycosyl, nucleosyl, lipoyl or R_1 is an L- or D- α amino acid or an oligomer thereof consisting of up to 5 residues R_1 is absent when the amino acid adjacent is a desamino-derivative.

35 R_2 is $-NR_{12}R_{13}$, wherein R_{12} and R_{13} are

independently H, straight, branched or cyclic alkyl,
aralkyl or aryl optionally substituted as defined for R₁
or N-glycosyl or N-lipoyl -OR₁₄, where R₁₄ is H,
straight, branched or cyclic alkyl, aralkyl or aryl,
5 optionally substituted as defined for R₁-O-glycosyl,
-O-lipoyl or - an L- or D- α -amino acid or an oligomer
thereof consisting of up to 5 residues or R₂ is absent,
when the adjacent amino acid is a decarboxy derivative of
cysteine or a homologue thereof or the peptide is in a N-C
10 cyclic form.

In a preferred embodiment of the second aspect of the
present invention the peptide is Gly-Ala-Gln-Met-Cys(Acm)-
Cys(Acm)-Ser-Lys-Cys(Acm)-Ser-Pro-Gly-Gln-His-Gly.

The amino acids may be D or L isomers, however,
15 generally the peptide will primarily consist of L-amino
acids. In addition, the cysteine residues may also
include an Acn group protecting the cysteine residues.

In a third aspect the present invention consists in a
pharmaceutical composition for use in treating subjects
20 suffering from TNF toxicity, the composition comprising a
peptide of the first or second aspect of the present
invention in combination with a pharmaceutically
acceptable sterile carrier.

In a fourth aspect the present invention consists in
25 a method of treating a subject suffering from the toxic
effects of TNF, the method comprising administering to the
subject the composition of the third aspect of the present
invention.

In a preferred embodiment of the present invention
30 the peptide is Peptide 371 as hereinafter defined.

The peptide of the present invention may be used in
therapy to prevent TNF pathology associated with decreased
blood glucose levels and weight loss and may be a useful
therapy in the treatment of septic shock.

35 Further the composition and method of the present

inv ntion would be expected to be useful as an
anti-inflammatory agent in a wide range of disease states
including toxic shock, adult respiratory distress
syndrome, hypersensitivity pneumonitis, systemic lupus
5 erythromatosis, cystic fibrosis, asthma, bronchitis, drug
withdrawal, schistosomiasis, sepsis, rheumatoid arthritis,
acquired immuno-deficiency syndrome, multiple sclerosis,
leperosy, malaria, systemic vasculitis, bacterial
meningitis, cachexia, dermatitis, psoriasis, diabetes,
10 neuropathy associated with infection or autoimmune
disease, ischemia/reperfusion injury, encephalitis,
Guillame Barre Syndrome, atherosclerosis, chronic fatigue
syndrome, TB, other viral and parasitic diseases, OKT3
therapy, and would be expected to be useful in conjunction
15 with radiation therapy, chemotherapy and transplantation,
to ameliorate the toxic effects of such treatments or
procedures.

As the peptide of the present invention suppresses
activation of neutrophils the composition and method of
20 the present invention may also be useful in the treatment
of diseases with an underlying element of local, systemic,
acute or chronic inflammation. In general, it is believed
the composition and method of the present invention will
be useful in treatment of any systemic or local infection
25 leading to inflammation.

The peptides of the present invention may also be
administered in cancer therapy in conjunction with
cytotoxic drugs which may potentiate the toxic effects of
TNF α (Watanabe et al., 1988; Immunopharmacol.
30 Immunotoxicol. 10: 117-127) such as vinblastin, acyclovir,
interferon alpha, cyclosporin A, IL-2, actinomycin D,
adriamycin, mitomycin C, AZT, cytosine arabinoside,
daunororubin, cis-platin, vincristine, 5-flurouracil and
bleomycin; in cancer patients undergoing radiation
35 therapy; and in AIDS patients (or others suffering from

viral infection such as viral meningitis, hepatitis, herpes, green monkey virus etc.) and in patients receiving immunostimulants such as thymopentin and muramyl peptides or cytokines such as IL-2 and GM-CSF. In this use
5 peptides of the present invention will serve to abrogate the deleterious effects of $TNF\alpha$.

It will be appreciated by those skilled in the art that a number of modifications may be made to the peptide of the present invention without deleteriously effecting
10 the biological activity of the peptide. This may be achieved by various changes, such as insertions, deletions and substitutions (e.g., sulfation, phosphorylation, nitration, halogenation), either conservative or non-conservative (e.g., W-amino acids, desamino acids) in
15 the peptide sequence where such changes do not substantially altering the overall biological activity of the peptide. By conservative substitutions the intended combinations are:-

G, A; V, I, L, M; D, E; N, Q; S, T; K, R, H;
20 F, Y, W, H; and P, $N\alpha$ -alkylamino acids.

It may also be possible to add various groups to the peptide of the present invention to confer advantages such as increased potency or extended half-life in vivo, without substantially altering the overall biological
25 activity of the peptide.

The term peptide is to be understood to embrace peptide bond replacements and/or peptide mimetics, i.e. pseudopeptides, as recognised in the art (see for example: Proceedings of the 20th European Peptide Symposium, edt.
30 G. Jung. E. Bayer, pp. 289-336, and references therein), as well as salts and pharmaceutical preparations and/or formulations which render the bioactive peptide(s) particularly suitable for oral, topical, nasal spray, ocular pulmonary, I.V., subcutaneous, as the case may be,
35 delivery. Such salts, formulations, amino acid

replacements and pseudopeptide structures may be necessary and desirable to enhance the stability, formulation, deliverability (e.g., slow release, prodrugs), or to improve the economy of production, and they are acceptable, provided they do not negatively affect the required biological activity of the peptide.

Apart from substitutions, three particular forms of peptide mimetic and/or analogue structures of particular relevance when designating bioactive peptides, which have to bind to a receptor while risking the degradation by proteinases and peptidases in the blood, tissues and elsewhere, may be mentioned specifically, illustrated by the following examples: Firstly, the inversion of backbone chiral centres leading to D-amino acid residue structures may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation while not impairing activity. An example is given in the paper "Tritiated D-ala¹-Peptide T Binding", Smith, C.S. et al, Drug Development Res. 15, pp. 371-379 (1988). Secondly, cyclic structure for stability, such as N to C interchain imides and lactames (Ede et al in Smith and Rivier (Eds) "Peptides: Chemistry and Biology", Escom, Leiden (1991), p268-270), and sometimes also receptor binding may be enhanced by forming cyclic analogues. An example of this is given in "Conformationally restricted thymopentin-like compounds", U.S. pat. 4,457,489 (1985), Goldstein, G. et al. Finally, the introduction of ketomethylene, methylsulfide or retroinverse bonds to replace peptide bonds, i.e. the interchange of the CO and NH moieties may both greatly enhance stability and potency. An example of the latter type is given in the paper "Biologically active retroinverso analogues of thymopentin", Sisto A. et al in Rivier, J.E. and Marshall, G.R. (eds.) "Peptides, Chemistry, Structure and Biology", Escom, Leiden (1990), p.722-773.

The peptides of the invention can be synthesized by various methods which are known in principle, namely by chemical coupling methods (cf. Wunsch, E.: "Methoden der organischen Chemie", Volume 15, Band 1 + 2, Synthese von Peptiden, Thieme Verlag, Stuttgart (1974), and Barrany, G.; Merrifield, R.B: "The Peptides", eds. E. Gross, J. Meienhofer., Volume 2, Chapter 1, pp. 1-284, Academic Press (1980)), or by enzymatic coupling methods (cf. Widmer, F., Johansen, J.T., Carlsberg Res. Commun., Volume 44, pp. 37-46 (1979), and Kullmann, W.: "Enzymatic Peptide Synthesis", CRC Press Inc., Boca Raton, Florida (1987), and Widmer, F., Johansen, J.T. in "Synthetic Peptides in Biology and Medicine:", eds., Alitalo, K., Partanen, P., Vattieri, A., pp. 79-86, Elsevier, Amsterdam (1985)), or by a combination of chemical and enzymatic methods if this is advantageous for the process design and economy.

It will be seen that one of the alternatives embraced in the general formula set out above is for a cysteine residue to be positioned at both the amino and carboxy terminals of the peptide. This will enable the cyclisation of the peptide by the formation of di-sulphide bond.

It is intended that such modifications to the peptide of the present invention which do not result in a decrease in biological activity are within the scope of the present invention.

As would be recognized by those skilled in the art there are numerous examples to illustrate the ability of anti-idiotypic (anti-Ids) antibodies to an antigen to function like that antigen in its interaction with animal cells and components of cells. Thus, anti-Ids to a peptide hormone antigen can have hormone-like activity and interact specifically with the receptors to the hormone. Conversely, anti-Ids to a receptor can interact specifically with a mediator in the same way as the

receptor does. (For a review of these properties see:
Gaulton, G.N. and Greane, M.I. 1986. Idiotypic mimicry of
biological receptors, Ann. Rev. Immunol. 4, 253-280;
Sege, K and Peterson, P.A., 1978. Use of anti-idiotypic
5 antibodies as cell surface receptor probes. Proc. Natl.
Acad. Sci. U.S.A. 75, 2443-2447).

As might be expected from this functional similarity
of anti-Id and antigen, anti-Ids bearing the internal
image of an antigen can induce immunity to such an
10 antigen. (This nexus is reviewed in Hiernaux, J.R. 1988.
Idiotypic vaccines and infectious diseases. Infect.
Immun. 56, 1407-1413.)

As will be appreciated by persons skilled in the art
from the disclosure of this application it will be
15 possible to produce anti-idiotypic antibodies to the
peptide of the present invention which will have similar
biological activity. It is intended that such
anti-idiotypic antibodies are included within the scope of
the present invention.

20 Accordingly, in a fifth aspect the present invention
consists in an anti-idiotypic antibody to the peptide of
the first aspect of the present invention, the
anti-idiotypic antibody being capable of reducing TNF
toxicity.

25 The individual specificity of antibodies resides in
the structures of the peptide loops making up the
Complementary Determining Regions (CDRs) of the variable
domains of the antibodies. Since in general, the amino
acid sequences of the CDR peptide loops of an anti-Id are
30 not identical to or even similar to the amino acid
sequence of the peptide antigen from which it was
originally derived, it follows that peptides whose amino
acid sequence is quite dissimilar, in certain contexts can
take up a very similar three-dimensional structure. The
35 concept of this type of peptide, termed a "functionally

equivalent sequence" or mimotope by Geyson is familiar to those expert in the field. (Geyson, H.M. et al 1987. Strategies for epitope analysis using peptide synthesis. J. Immun. Methods. 102, 259-274).

5 Moreover, the three-dimensional structure and function of the biologically active peptides can be simulated by other compounds, some not even peptidic in nature, but which mimic the activity of such peptides. This field of science is summarised in a review by
10 Goodman, M. (1990). (Synthesis, spectroscopy and computer simulations in peptide research. Proc. 11th American Peptide Symposium published in Peptides-Chemistry, Structure and Biology pp 3-29. Ed Rivier, J.E. and Marshall, G.R. Publisher ESCOM.)

15 As will be recognized by those skilled in the art, armed with the disclosure of this application, it will be possible to produce peptide and non-peptide compounds having the same three-dimensional structure as the peptide of the present invention. These "functionally equivalent
20 structures" or "peptide mimics" will react with antibodies raised against the peptide of the present invention and may also be capable of reducing TNF toxicity. It is intended that such "peptide mimics" are included within the scope of the present invention.

25 Accordingly, in a sixth aspect the present invention consists in a compound the three-dimensional structure of which is similar as a pharmacophore to the three-dimensional structure of the peptide of the first or second aspect of the present invention, the compound being
30 characterized in that it reacts with antibodies raised against the peptide of the first aspect of the present invention and that the compound is capable of reducing TNF toxicity.

More detail regarding pharmacophores can be found in
35 Rolin et al. p 150, Polinsky et al. p 287, and Smith et

al. p 485 in Smith and Rivier (Eds) "Peptides: Chemistry and Biology", Escom, Leiden (1991).

Detailed Description of the Invention

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples and Figures in which:-

Figure 1 shows changes in blood glucose levels in ascites tumour-bearing mice in response to TNF treatment
 10 □ 0 hrs, ■ 24 hrs; and

Figure 2 shows weight loss by tumour-bearing mice as a result of TNF treatment; and

Figure 3 shows the effect of peptides on TNF-induced lethality, ■ 24 hrs post TNF; □ 30 hrs post TNF.

15 Peptide Synthesis

Peptides numbered 363, 369-374 listed below were synthesized either by the Boc or Fmoc strategies.

(Abbreviations used in the following description: AcM Acetamidomethyl, OCxl Cyclohexyl ester, Pmc

20 2,2,5,7,8-Pentamethylchroman-6-sulfonyl, NMM N-Methylmorpholine).

363 H-Pro-Gln-Gly-Lys-Tyr-OH

369 H-Arg-Asp-Thr-Val-Cys(AcM)-Gly-Cys(AcM)-Arg-Lys-Asn-Gln
 -Tyr-Arg-His-OH

25 370 Ac-Gln-Asp-Thr-Asp-Cys(AcM)-Arg-Glu-Cys(AcM)-Glu-Ser-
 Gly-Ser-Phe-Thr-Ala-Ser-Glu-Asn-His-Leu-Arg-His-OH

371 H-Leu-Tyr-Asn-Asp-Cys(AcM)-Pro-Gly-Pro-Gly-Gln-Asp-Thr
 -Asp-OH

372 H-Asp-Ser-Val-Cys(AcM)-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-
 30 Gln-Asn-Asn-Ser-OH

373 H-Thr-Lys-Cys(AcM)-His-Lys-Gly-Thr-OH

374 H-Glu-Asn-Val-Lys-Gly-Thr-Glu-Asp-Ser-Gly-Thr-Thr-OH

466 Leu-Tyr-Asn-Asp-Cys(AcM)-Pro-Gly-Pro-Gln

35 Asp-Thr-Asp-Cys(AcM)-Arg-Glu-Cys(AcM)-Glu-Ser-Gly-Ser-
 Phe-Thr

- 467 Thr-Lys-Cys(Acm)-His-Lys-Gly-Thr-Tyr-Leu-Tyr-Asn-Asp-
Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp
- 536 Arg-Glu-Asn-Glu-Cys(Acm)-Val-S r-Cys(Acm)-Ser-Asn-
Cys(Acm)-Thr-Lys-Leu
- 5 630 Ac-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly
631 Gly-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly
632 Gly-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly
633 Gly-Pro-Gly-Gln-Asp-Thr-Asp
634 Ac-Gly-Pro-Gly-Gln-Asp-Thr-Asp-NH₂
- 10 635 Gly-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Gly
636 Ac-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-
NH₂
- 637 Gly-Ala-Gln-Met-Cys(Acm)-Cys(Acm)-Ser-Lys-Cys(Acm)-
Ser-Pro-Gly-Gln-His-Gly

15 SYNTHESIS OF PEPTIDES USING THE FMOC-STRATEGY

Peptides were synthesized either on the continuous flow system using the standard Fmoc-polyamide method of solid phase peptide synthesis (Atherton et al, 1978, J. Chem. Soc. Chem. Commun., 13, 537-539) or alternatively on the stirred cell system using polystyrene based resins.

For peptides with free carboxyl at the C-terminus, the solid resin used was PepSyn KA which is a polydimethylacrylamide gel on Kieselguhr support with 4-hydroxymethylphenoxyacetic acid as the functionalised linker (Atherton et al., 1975, J.Am.Chem.Soc. 97, 6584-6585). The carboxy terminal amino acid was attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification. The alternative is the Wang resin with Fmoc-amino acid previously attached.

For peptides with carboxamides at the C-terminus, the solid resin used was Fmoc-PepSyn K Am which is analogous polyamide resin with a Rink Linker, p-[(R,S)- α [1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (Bernatowicz et al, 1989, Tet. Lett. 30, 4645). The synthesis starts by removing the

Fmoc-group with an initial piperidine wash and incorporation of the first amino acid is carried out by the usual peptide coupling procedure. In the stirred cell system, this is replaced by the Rink resin which is a polystyrene-based support.

All Fmoc-groups during synthesis were removed by 20% piperidine/DMF and peptide bonds were formed either of the following methods except as indicated in Table 1:

1. Pentafluorophenyl active esters - the starting Fmoc amino acids are already in the active ester form.
2. Hydroxybenzotriazol esters - these are formed in situ either using Castro's reagent, BOP/NMM/HOBt (Fournier et al, 1989, Int. J. Peptide Protein Res., 33, 133-139) or using Knorr's reagent, HBTU/NMM/HOB-t (knorr et al, 1989, Tet. Lett., 30, 1927)

Side chain protection chosen for the amino acids was removed concomitantly during cleavage with the exception of Acn on cysteine which was left on after synthesis.

TABLE 1

<u>20</u>	<u>Amino Acid</u>	<u>Protecting Group</u>	<u>Coupling Method</u>
	Arg	Pmc	HOBt or OPfp
	Asp	OBu	HOBt or OPfp
	Cys	Acn	HOBt or OPfp
	Glu	OBu	HOBt or OPfp
<u>25</u>	His	Boc or Trt	HOBt or OPfp
	Lys	Boc	HOBt or OPfp
	Ser	But	HOBt only
	Thr	But	HOBt only
	Tyr	But	HOBt or OPfp
<u>30</u>	Asn	none	OPfp only
		Trt	HOBt or OPfp
	Gln	none	OPfp only
		Trt	HOBt or OPfp

Cleavage Conditions

Peptides were cleaved from the PepSyn KA and PepSyn K Am using 5% water and 95% TFA where Arg(Pmc) is not present. Where Arg(Pmc) is present a mixture of 5% thioanisole in TFA is used. The cleavage typically took 3 h at room temperature with stirring. Thioanisole was removed by washing with ether or ethyl acetate and the peptide was extracted into an aqueous fraction. Up to 30% acetonitrile was used in some cases to aid dissolution. Lyophilization of the aqueous/acetonitrile extract gave the crude peptide.

Peptides from the Wang resin were cleaved in TFA containing 5% phenol, for up to 2 h at ambient temperature with stirring.

15 N-TERMINAL ACETYLTATION

The peptide resin obtained after the synthesis (with Fmoc removed in the usual manner) was placed in a 0.3 M DMF solution of 10 equivalents of Ac-ONSu or acetic anhydride for 60 minutes. The resin was filtered, washed with DMF, CH_2Cl_2 , ether and dried.

SYNTHESIS OF PETIOIDES USING THE BOC-STRATEGY

Syntheses of these peptides were carried out using polystyrene based resins. For peptide with C-terminal acids, the appropriate Merrifield resin Boc-amino acid-O-resin or the 100-200 mesh PAM resin is used. Peptides with C-terminal amides are synthesized on MBHA resins.

Couplings of Boc-amino acids (Table 2) were carried out either using Symmetrical anhydride method or a HOBt ester method mediated by DCC or HTBU.

TABLE 2

	<u>Amino Acid</u>	<u>Protecting Group</u>	<u>Coupling Method/Reagent</u>
	Arg	Tos	HOBt or S.A.
	Asp	OCxl, OBzl	HOBt or S.A.
5	Cys	4-MeBzl	HOBt or S.A.
	Glu	OCxl	HOBt or S.A.
	His	Dnp, Bom	HOBt or S.A.
	Lys	2-ClZ	HOBt or S.A.
	Ser	Bzl	HOBt or S.A.
10	Thr	Bzl	HOBt or S.A.
	Tyr	Br-Z	HOBt or S.A.
	Asn	Xan	HOBt or S.A.
	Gln	none	HOBt only

CLEAVAGE

- 15 Peptides were cleaved in HF with p-cresol or anisole as scavenger for up to 90 min. For His with Dnp protection, the resin required pre-treatment with mercaptoethanol:DIPEA:DMF (2:1:7), for 30 min. After removal of scavengers by ether wash, the crude peptide is
- 20 extracted into 30% aqueous acetonitrile.

N-TERMINAL ACETYLTATION

Acetylation was achieved by treating the deblocked resin with acetic anhydride in DMF solution.

PURIFICATION OF PEPTIDES

- 25 Crude peptide is purified by reverse phase chromatography using either a C4 or C18 column and the Buffer system: Buffer A - 0.1% aqueous TFA, Buffer B - 80% Acetonitrile and 20% A. Fractions are monitored by an analytical h.p.l.c. system with a diode array detection.
- 30 Structures were confirmed by amino acid analysis, proton n.m.r. and FAB mass spectrometry.

Example 1

371 H-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-A
sp-OH

- 35 The resin Boc-Asp (Cxl)-O-Resin (100-200 mesh

0.75 mM/g) was used in the synthesis. This is followed by the coupling of the subsequent residues in the following order:

	Boc-Thr(Bzl)-OH	DCC	S.A. (Symmetrical anhydride)
5	Boc-Asp-(OCxl)-OH	DCC	S.A.
	Boc-Gln-OH	DCC/HOBt	
	Boc-Gly-OH	DCC	S.A.
	Boc-Pro-OH	DCC	S.A.
	Boc-Gly-OH	DCC	S.A.
10	Boc-Pro-OH	DCC	S.A.
	Boc-Cys(Acm)-OH	DCC/HOBt	
	Boc-Asp(OCxl)-OH	DCC	S.A.
	Boc-Asn-OH	DCC/HOBt	
	Boc-Tyr(2Br-Z)	DCC	S.A.
15	Boc-Leu-OH	DCC	S.A.

The peptide resin (1.5 g) was treated with 15 ml HF at 0°C for 60 min. in the presence of p-cresol (1.5 g). The peptide resin was washed with diethyl ether and then extracted with 30% aqueous acetonitrile. The peptide collected in the aqueous fraction was lyophilized to give 570 mg.

Purification was carried out in a Waters Delta Prep instrument using a Delta Pak, C18 Prep-Pak cartridge, 300 A, 15 µm. Buffer A: 0.1% TFA/water and buffer B is 90% acetonitrile/water (0.1% TFA). The gradient is 0-40% B over 60 min. (linear) monitored at 230 nm at a flow rate of 20m./min. A yield of 90 mg of 94% pure peptide was obtained.

Amino acid analysis gave Asp 4.0 (4.0) Glu 1.1(1.0) Gly 2.0(2.0) Leu 1.0(1.0) Pro 1.9(2.0) Thr 1.0(1.0) Tyr 0.7(1.0) and Cys was present.

Fast atom bombardment mass spectroscopy using argon gas gave a M+H peak at 1465.

IV Example 2

35 374 H-Glu-Asn-Val-Lys-Gly-Thr-Glu-Asp-Ser-Gly-Thr-Thr-OH

Pepsyn KA (2.0 g, 0.25 mMol/g) was esterified with Fmoc-Thr-OH using 8 equiv of Fmoc-Thr(But)-OH, 4 equiv of DCC and 0.4 equiv of DMAP.

The following amino acid derivatives and coupling conditions were used in subsequent steps:

5	Fmoc-Thr(But)-OH	BOP/HOBt/NMM
	Fmoc-Gly-OH	BOP/HOBt/NMM
	Fmoc-Ser(But)-OH	BOP/HOBt/NMM
	Fmoc-Asp(OBut)-OH	BOP/HOBt/NMM
10	Fmoc-Glu(OBut)-OH	BOP/HOBt/NMM
	Fmoc-Thr(But)-OH	BOP/HOBt/NMM
	Fmoc-Gly-OH	BOP/HOBt/NMM
	Fmoc-Lys(Boc)-OH	BOP/HOBt/NMM
	Fmoc-Val-OH	BOP/HOBt/NMM
15	Fmoc-Asn-OPfp	-
	Fmoc-Glu(OBut)-OH	BOP/HOBt/NMM

The peptide resin, after removal of the final Fmoc group weighed 2.76 g. Cleavage was carried in 95% aqueous TFA and the crude peptide, after lyophilization gave
20 813 mg.

The crude peptide was purified on a Waters' Delta Prep using a C18 Prep Pak cartridge 300 A 15 μ m particle size. Buffer A is 0.1% TFA and Buffer B is 80% Acetonitrile and 20% buffer A and a linear gradient of
25 0-80%B over 40 min monitored at 23 nm with a flow rate of 30 ml/min, gave 262 mg of the 90% pure peptide.

Amino acid analysis gave Asp 1.9(2.0) Glu 2.1(2.0) Ser 0.9(1.0) Gly 2.3(2.0) Thr 2.9(3.0) Val 1.0(1.0) Lys 1.0(1.0). FAB mass spectrum of the peptide gives a M+H
30 peak at 1237.

In Vivo Experiments

Balb/c mice (female, aged 10-12 weeks) were inoculated with Meth A ascites tumour cells (2×10^6) two weeks before treatment with TNF (10 μ g) alone or in
35 combination with TNF inhibiting peptide (10 mg, Fig. 1).

At the time of treatment and 24 hrs after treatment measurements were taken of weight and blood glucose levels. Figure 1 shows changes in blood glucose levels in ascites tumour-bearing mice in response to TNF treatment. It can be seen that peptide 371 inhibited the TNF-induced decrease in blood glucose levels which occurred within the 24 hours following TNF treatment. Peptide 371 also inhibited weight loss by the tumour-bearing animals as a result of TNF treatment (Figure 2).

In lethality experiments Balb/C mice (female, aged 10 - 12 weeks) were primed intraperitoneally with pristane (0.5 ml) 10 days prior to peritoneal implantation of MethA tumour cells. Approximately 10 days later the mice were challenged with 25µg recombinant human TNF administered subcutaneously and the number of mice surviving at 24 and 30 hours recorded. Mice were also treated with peptide 1 mg or anti-TNF monoclonal antibody (M47) by inoculation at a separate subcutaneous site. The results of these experiments are shown in Figure 3 and Table 3.

20

TABLE 3

EFFECT OF PEPTIDE 371 ON SURVIVAL OF MICE ADMINISTERED A LETHAL DOSE OF TNF

	<u>TREATMENT</u>	<u>NO./GROUP</u>	<u>NO. SURVIVORS*</u>
25	PBS	10	0
	M47	10	7
	371	6	2

* 24 hours post TNF treatment

30

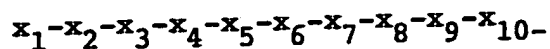
As will be seen from the above results the peptides of the present invention, and in particular peptide 371, are capable of preventing a decrease in blood glucose

levels and weight loss in tumour-bearing mice treated with human recombinant tumour necrosis factor. In the light of these results it is believed these peptides have utility in the treatment of numerous disease states which are due
5 to the deleterious effects of TNF.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the
10 invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

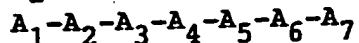
CLAIMS:

1. A linear or cyclic peptide of the general formula:-



in which x_1 is null, Cys or R_1 ;

5 x_2 is null, Cys, R_1 or



in which A_1 is Ser or Thr or Ala,

A_2 is Lys or Arg or His,

A_3 is Cys or Arg or His,

10 A_4 is His or Lys or Arg or Phe or Tyr or Trp,

A_5 is Lys or Arg or His,

A_6 is Gly or Ala,

A_7 is Thr or Ser or Ala,

x_3 is Null, Cys, R_1 or A_8-A_9

15 in which A_8 is absent or Gly or Ala or Tyr or Phe or Trp or His

A_9 is Leu or Ile or Val or Met

x_4 is Cys, R_1 or

$A_{10}-A_{11}-A_{12}-A_{13}-A_{14}$ in which A_{10} is

20 Tyr or Phe or Trp or His or Gly or Ala,

A_{11} is Asn or Gln,

A_{12} is Asp or Glu,

A_{13} is Cys or Arg or His,

A_{14} is Pro or $N\alpha$ - alkyl amino acid

25 x_5 is Gly or Ala,

x_6 is Cys, R_2 or $A_{15}-A_{16}$

in which A_{15} is Pro or $N\alpha$ - alkyl amino acid, A_{16} is Gly or Ala,

x_7 is null, Cys, R_2 or $A_{17}-A_{18}-A_{19}$

30 in which A_{17} is Gln or Asn,

A_{18} is Asp or Glu,

A_{19} is Thr or Ser or Ala,

x_8 is null, Cys, R_2 , Asp, Glu, Gly or Ala,

x_9 is null, Cys, R_2 or

35 $A_{20}-A_{21}-A_{22}-A_{23}-A_{24}-A_{25}-A_{26}-A_{27}$

-A₂₈-A₂₉

- in which A₂₀ is Cys or Arg or His,
 A₂₁ is Arg or Lys or His,
 A₂₂ is Glu or Asp,
 5 A₂₃ is Cys or Arg or His,
 A₂₄ is Glu or Asp,
 A₂₅ is Ser or Thr or Ala,
 A₂₆ is Gly or Ala,
 A₂₇ is Ser or Thr or Ala,
 10 A₂₈ is Phe or Tyr or Trp or His,
 A₂₉ is Thr or Ser or Ala,
 X₁₀ is null, Cys or R₂

- R₁ is R-CO, where R is H, straight, branched or cyclic alkyl up to C20, optionally containing double bonds and/or
 15 substituted with halogen, nitro, amino, hydroxy, sulfo, phospho or carboxyl groups (which may be substituted themselves), or aralkyl or aryl optionally substituted as listed for the alkyl and further including alkyl, or R₁ is glycosyl, nucleosyl, lipoyl or R₁ is an L- or D- α
 20 amino acid or an oligomer thereof consisting of up to 5 residues R₁ is absent when the amino acid adjacent is a desamino-derivative.

- R₂ is -NR₁₂R₁₃, wherein R₁₂ and R₁₃ are independently H, straight, branched or cyclic alkyl,
 25 aralkyl or aryl optionally substituted as defined for R₁ or N-glycosyl or N-lipoyl -OR₁₄, where R₁₄ is H, straight, branched or cyclic alkyl, aralkyl or aryl, optionally substituted as defined for R₁-O-glycosyl, -O-lipoyl or - an L- or D- α -amino acid or an oligomer
 30 thereof consisting of up to 5 residues or R₂ is absent, when the adjacent amino acid is a decarboxy derivative of cysteine or a homologue thereof or the peptide is in a N-C cyclic form.

with the proviso that:

- 35 X₁ is always and only null when X₂ is R₁, Cys or null

- X_2 is always and only null when X_3 is R_1 , Cys or null
 X_3 is always and only null when X_4 is R_1 or Cys
 X_7 is always and only null when X_6 is R_2 or Cys
 X_8 is always and only null when X_7 is null, R_2 or Cys
 5 X_9 is always and only null when X_8 is null, R_2 or Cys
 X_{10} is always and only null when X_9 is null, R_2 or Cys

when X_4 is R_1 or Cys then X_6 is $A_{15}-A_{16}$, X_7 is
 $A_{17}-A_{18}-A_{19}$ and X_8 is Asp, Glu, Gly or Ala,

- 10 when X_6 is R_2 or Cys then X_4 is

$A_{10}-A_{11}-A_{12}-A_{13}-A_{14}$

when X_7 is null, R_2 or Cys then X_4 is

$A_{10}-A_{11}-A_{12}-A_{13}-A_{14}$.

2. A linear or cyclic peptide as claimed in claim 1 in
 15 which the peptide is selected from the group consisting
 of:-

H-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-
 Asp-OH,

- 20 Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gln-Asp-Thr-Asp-
 Cys(Acm)-Arg-Glu-Cys(Acm)-Glu-Ser-Gly-Ser-Phe-Thr,

Thr-Lys-Cys(Acm)-His-Lys-Gly-Thr-Tyr-Leu-Tyr-Asn-Asp-
 Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp,

Ac-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly,

Gly-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly,

- 25 Gly-Leu-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly,

Gly-Pro-Gly-Gln-Asp-Thr-Asp,

Ac-Gly-Pro-Gly-Gln-Asp-Thr-Asp-NH₂,

Gly-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Gly, and

Ac-Tyr-Asn-Asp-Cys(Acm)-Pro-Gly-Pro-Gly-Gln-Asp-Thr-NH₂

- 30 3. A linear or cyclic peptide as claimed in claim 1 in
 which the peptide is;

H-Leu-Tyr-Asn-Asp-Cys-Pro-Gly-Pro-Gly-Gln-Asp-Thr-Asp-OH.

4. A linear or cyclic peptide of the general formula:-

$Y_1-Y_2-Y_3-Y_4-Y_5-Y_6-Y_7-Y_8-Y_9-Y_{10}-$

- 35 $Y_{11}-Y_{12}-Y_{13}-Y_{14}-Y_{15}-Y_{16}-Y_{17}$

- in which Y_1 is null, Cys or R_1
 Y_2 is Gly or Ala
 Y_3 is Ala or Gla
 Y_4 is Gln or Asn
5 Y_5 is Met or Gal or Ile or Leu
 Y_6 is Cys or Arg or His
 Y_7 is Cys or Arg or His
 Y_8 is Ser or Thr or Ala
 Y_9 is Lys or Arg or His
10 Y_{10} is Cys or Arg or His
 Y_{11} is Ser or Thr or Ala
 Y_{12} is Pro or α - alkyl amino acid
 Y_{13} is Gly or Ala
 Y_{14} is Gln or Asn
15 Y_{15} is His or Lys or Arg
 Y_{16} is Gly or Ala
 Y_{17} is null, Cys or A_2
 R_1 is R-CO, where R is H, straight, branched or cyclic
alkyl up to C20, optionally containing double bonds and/or
20 substituted with halogen, nitro, amino, hydroxy, sulfo,
phospho or carboxyl groups (which may be substituted
themselves), or aralkyl or aryl optionally substituted as
listed for the alkyl and further including alkyl, or R_1
is glycosyl, nucleosyl, lipoyl or R_1 is an L- or D- α
25 amino acid or an oligomer thereof consisting of up to 5
residues R_1 is absent when the amino acid adjacent is a
desamino-derivative.
 R_2 is $-NR_{12}R_{13}$, wherein R_{12} and R_{13} are
independently H, straight, branched or cyclic alkyl,
30 aralkyl or aryl optionally substituted as defined for R_1
or N-glycosyl or N-lipoyl $-OR_{14}$, where R_{14} is H,
straight, branched or cyclic alkyl, aralkyl or aryl,
optionally substituted as defined for R_1 -O-glycosyl,
-O-lipoyl or - an L- or D- α -amino acid or an oligomer
35 thereof consisting of up to 5 residues or R_2 is absent,

when the adjacent amino acid is a decarboxy derivative of cysteine or a homologue thereof or the peptide is in a N-C cyclic form.

5. A linear or cyclic peptide as claimed in claim 4 in which the peptide is;
5 Gly-Ala-Gln-Met-Cys(Acm)-Cys(Acm)-Ser-Lys-Cys(Acm)-Ser-Pro-Gly-Gln-His-Gly.

6. A pharmaceutical composition for use in treating subjects suffering from TNF toxicity, the composition
10 comprising a peptide as claimed in any one of claims 1 to 5 in combination with a pharmaceutically acceptable sterile carrier.

7. A method of treating a subject suffering from the toxic effects of TNF, the method comprising administering
15 to the subject the composition as claimed in claim 6.

8. A method as claimed in claim 7 in which the subject is suffering from TNF pathology associated with decreased blood glucose levels and/or weight loss or from septic shock.

20 9. An anti-idiotypic antibody to the peptide as claimed in any one of claims 1 to 5, the anti-idiotypic antibody being characterised in that it is capable of reducing TNF toxicity.

10. A compound the three-dimensional structure of which
25 is similar as a pharmacophore to the three-dimensional structure of the peptide as claimed in any one of claims 1 to 5, the compound being characterized in that it reacts with antibodies raised against the peptide as claimed in any one of claims 1 to 5 and that the compound is capable
30 of reducing TNF toxicity.

1/3

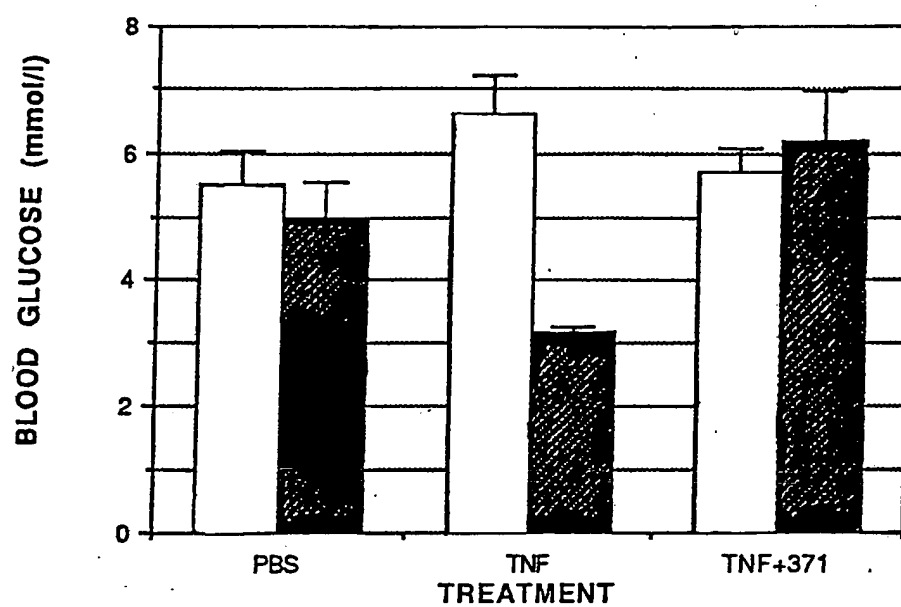


FIGURE 1.

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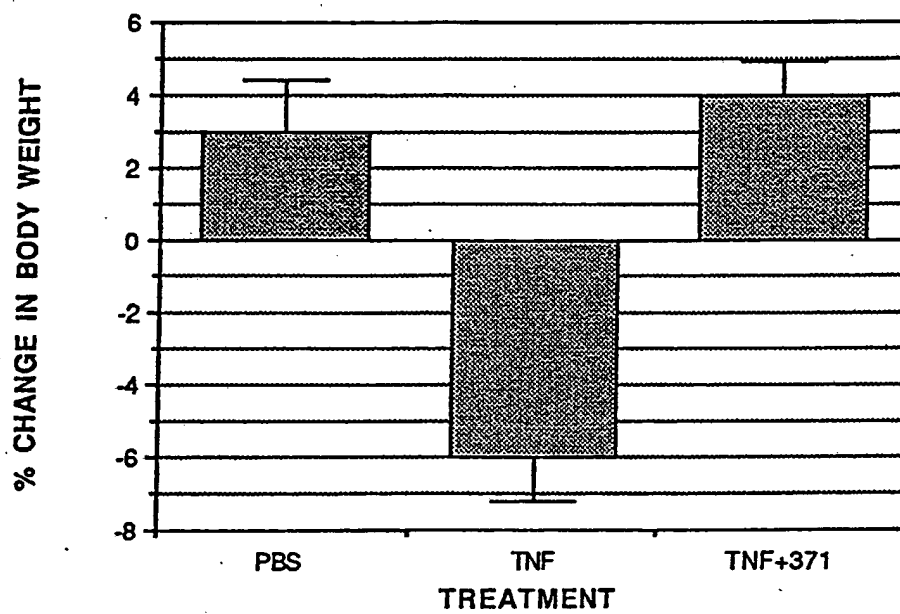


FIGURE 2.

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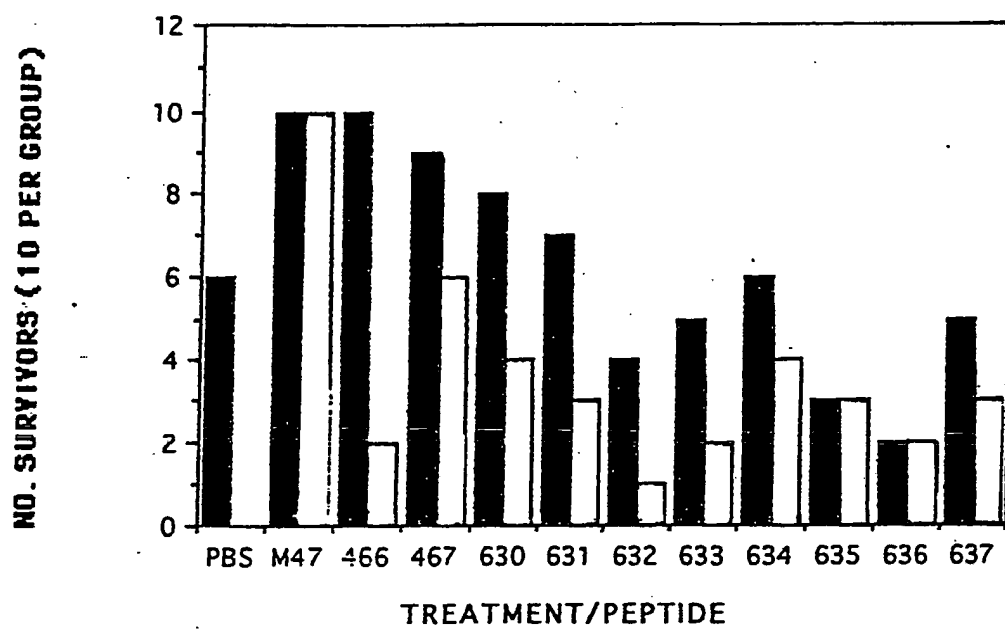
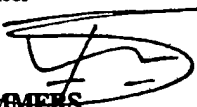


FIGURE 3.

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁵ C07K 7/06 7/08 7/10 7/64 A61K 37/02 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC C07K 7/06 7/08 7/10 7/64 A61K 37/02 C07C 103/52 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT: tumor necrosis or tumour necrosis or TNF or cachectin CHEMICAL ABSTRACTS PROTEIN SEQUENCE DATABASE				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.		
A	WO,A,91/03553 (IMMUNEX CORPORATION) 21 March 1991 (21.03.91) entire document, page 5, Figure 2	4-5		
X	Cell, Volume 61, Issued April 1990 Thomas J. Schall et. al. "Molecular cloning and Expression of a Receptor for human Tumor Necrosis Factor", pages 361-370. page 362 column 1, 4th last line and Figure 1A	1-3, 6-10		
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div> <input checked="" type="checkbox"/> See patent family annex. </div> </div>				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> * Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
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Date of the actual completion of the international search 15 December 1992 (15.12.92)		Date of mailing of the international search report 21 Dec. 1992 (24.12.92)		
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  TERRY SUMMERS Telephone No. (06) 2832291		

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	DNA and Cell Biology, volume 9, no. 10, 1990, Adolf Himmler et. al. "Molecular Cloning and Expression of Human and Rat Tumor Necrosis Factor Receptor Chain (p60) and Its Soluble Derivative, Tumor Necrosis Factor-Binding Protein", pages 705-715. page 709, Table 1 Fragment 14a and 20	1-3,6-10
A	GB,A,2246569 (THE CHARING CROSS SUNLEY RESEARCH CENTRE), 5 February 1992 (05.02.92) page 2 lines 11-24	1-3
A	Science, Volume 248, Issued May 1990, Craig A. Smith et. al. "A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins", pages 1019-1023 page 1021 Figure 3	1-3
A	The EMBO Journal, volume 9, no. 10, 1990 Yaron Nophar et. al. "Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type 1 TNF-R, cloned using amino and sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor", pages 3269-3278 entire document, page 3271 Figure 1D, page 3293 Figure 2	1-3
A	Cell, volume 61, Issued April 1990. Hansruedi Loetscher et. al. "Molecular Cloning and Expression of the Human 55kd Tumor Necrosis Factor Receptor", pages 351-359 page 353 Figure 2	1-3
A	Proc. Natl. Acad. Sci USA, Volume 87, Issued October 1990, Patrick W. Gray et. al. "Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein", pages 7380-7384 page 7382 Figure 1	1-3
A	Proc. Natl. Acad. Sci USA, Volume 87, Issued November 1990, Tadahiko Kohno et. al. "A second tumor necrosis factor receptor gene product can shed a naturally occurring tumour necrosis factor inhibitor", pages 8331-8335 page 8333 Figure 3	1-3
A	Molecular and Cell Biology, Volume 11, No. 6, Issued June 1991, Raymond G. Goodwin et al. "Molecular Cloning and Expression of the Type 1 and Type 2 Murine Receptors for Tumor Necrosis Factor", pages 3020-3026 page 3022 Figure 1B	1-3

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9103553	CA,A, 2065346 IL,A , 95572 NZ,A, 235148 ZA,A, 9007072	EP,A, 418014 JP,A, 3133382 AU,A, 6178/90	FI,A, 920946 NO,A, 920862 DD,A, 297664	
GB	2246569	GB,A, 9013410	GB,A, 2246569		
END OF ANNEX					

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/21, 31/40, 38/06, C07C 327/00, 327/36, 333/16, 333/20, 333/24, 333/32, C07D 207/04, C07K 5/09		A1	(11) International Publication Number: WO 95/30415 (43) International Publication Date: 16 November 1995 (16.11.95)
(21) International Application Number: PCT/US95/05880 (22) International Filing Date: 10 May 1995 (10.05.95) (30) Priority Data: 08/240,858 10 May 1994 (10.05.94) US 08/317,399 4 October 1994 (04.10.94) US (71) Applicant: EMORY UNIVERSITY [US/US]; 130 Oxford Road, Atlanta, GA 30332 (US). (72) Inventors: MEDFORD, Russell, M.; 2965 Cravey Trail, Atlanta, GA 30345 (US). ALEXANDER, R., Wayne; 453 Argonne Drive, Atlanta, GA 30305 (US). PARTHASARATHY, Sampath; 2958 Northbrook Drive, Atlanta, GA 30340 (US). KHAN, Bobby, V.; 5457 Brooke Ridge Drive, Dunwoody, GA 30338 (US). OFFERMANN, Margaret, K.; 2965 Garvey Trail, Atlanta, GA 30345 (US). (74) Agents: ZALESKY, Cheryl, K. et al.; Kilpatrick & Cody, Suite 2800, 1100 Peachtree Street, Atlanta, GA 30309-4530 (US).		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: TREATMENT FOR ATHEROSCLEROSIS AND OTHER CARDIOVASCULAR AND INFLAMMATORY DISEASES			
(57) Abstract Dithiocarboxylates, including dithiocarbamates, block the induced expression of the endothelial cell surface adhesion molecule VCAM-1, and are therefore useful in the treatment of cardiovascular disease, including atherosclerosis, as well as noncardiovascular inflammatory diseases that are mediated by VCAM-1.			

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**Treatment for Atherosclerosis
and Other Cardi vascular and Inflammatory Diseases**

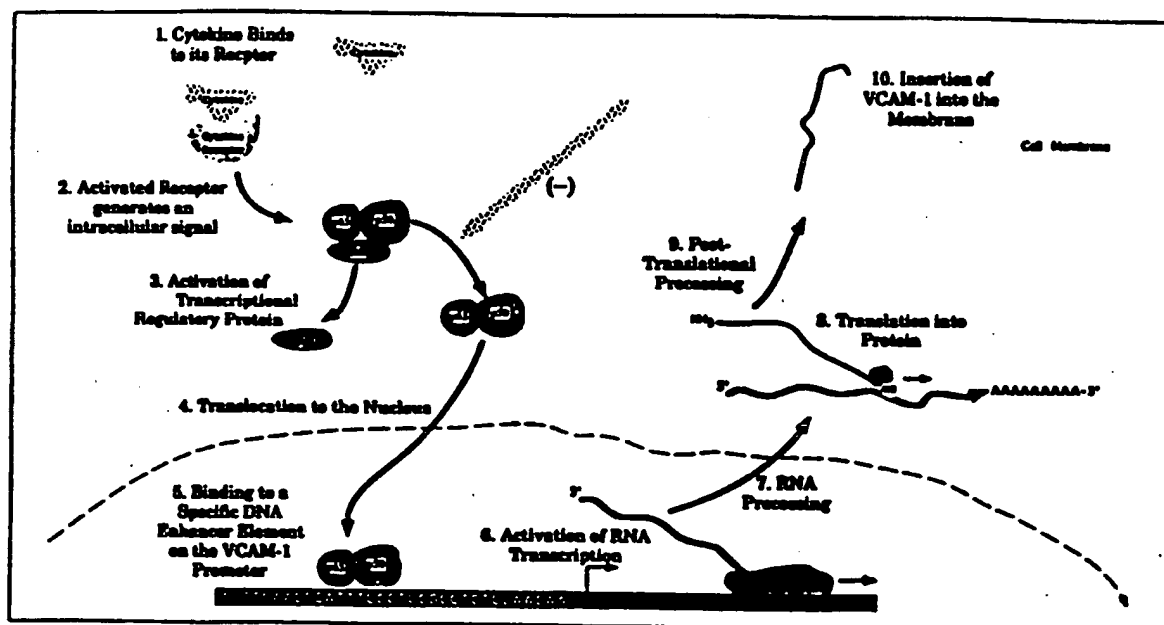
Backgr und of the Invention

This application is in the area of methods and compositions for the treatment of atherosclerosis and other cardiovascular and inflammatory diseases.

Adhesion of leukocytes to the endothelium
5 represents a fundamental, early event in a wide variety of inflammatory conditions, including atherosclerosis, autoimmune disorders and bacterial and viral infections. Leukocyte recruitment to the endothelium is started when inducible adhesion
10 molecule receptors on the surface of endothelial cells interact with counterreceptors on immune cells. Vascular endothelial cells determine which type of leukocytes (monocytes, lymphocytes, or neutrophils) are recruited, by selectively
15 expressing specific adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin. In the earliest stage of the atherosclerotic lesion, there is a localized
20 endothelial expression of VCAM-1 and selective recruitment of mononuclear leukocytes that express the integrin counterreceptor VLA-4. Because of the selective expression of VLA-4 on monocytes and lymphocytes, but not neutrophils, VCAM-1 is
25 important in mediating the selective adhesion of mononuclear leukocytes. Subsequent conversion of leucocytes to foamy macrophages results in the synthesis of a wide variety of inflammatory cytokines, growth factors, and chemoattractants
30 that help propagate the leukocyte and platelet recruitment, smooth muscle cell proliferation, endothelial cell activation, and extracellular matrix synthesis characteristic of maturing atherosclerotic plaque.

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VCAM-1 is expressed in cultured human vascular endothelial cells after activation by lipopolysaccharide (LPS) and cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF- α). These factors are not selective for activation of cell adhesion molecule expression. Scheme 1 illustrates the process of cytokine activation of VCAM-1 gene expression in vascular endothelial cells.



10

Scheme 1

Regulatory schemes for cytokine activation of vascular cell adhesion molecule-1 (VCAM-1) gene expression, through redox sensitive regulatory factors such as NF- κ B, in vascular endothelial cells (I κ B is an inhibitory subunit; NF- κ B is nuclear factor- κ B; NH₂ refers to the amino terminus of the protein and RNA Pol II is RNA polymerase II).

Molecular analysis of the regulatory elements on the human VCAM-1 gene that control its expression suggests an important role for nuclear factor- κ B (NF- κ B), a transcriptional regulatory factor, or an

-3-

NF- κ B like binding protein in oxidation-reduction-sensitive regulation of VCAM-1 gene expression. Transcriptional factors are proteins that activate (or repress) gene expression within the cell

5 nucleus by binding to specific DNA sequences called "enhancer elements" that are generally near the region of the gene, called the "promoter," from which RNA synthesis is initiated. Nuclear factor- κ B is a ubiquitously expressed multisubunit

10 transcription factor activated in several cell types by a large and diverse group of inflammatory agents such as TNF α , IL-1 β , bacterial endotoxin, and RNA viruses. It plays a key role in mediating inflammatory and other stress signals to the

15 nuclear regulatory apparatus. Although the precise biochemical signals that activate NF- κ B are unknown, this transcriptional factor may integrate into a common molecular pathway many of the risk factors and "causative" signals of atherosclerosis,

20 such as hyperlipidemia, smoking, hypertension, and diabetes mellitus.

Importantly, the activation of NF- κ B in vascular endothelial cells by diverse signals can be specifically inhibited by antioxidants such as

25 N-acetylcysteine and pyrrolidine dithiocarbamate (see U.S.S.N. 07/969,934, now allowed). This has led to the hypothesis that oxygen radicals play an important role in the activation of NF- κ B through an undefined oxidation-reduction mechanism.

30 Because an NF- κ B-like enhancer element also regulates the transcription of the VCAM-1 promoter in an oxidation-reduction-sensitive manner, oxidative stress in the atherosclerotic lesion may play a role in regulating VCAM-1 gene expression

35 through this oxidation-reduction-sensitive transcriptional regulatory protein.

It has been hypothesized that modification of low-density lipoprotein (LDL) into oxidatively modified LDL (ox-LDL) by reactive oxygen species is the central event that initiates and propagates atherosclerosis. Steinberg, et al., *N. Engl. J. Med.* 1989; 320:915-924. Oxidized LDL is a complex structure consisting of at least several chemically distinct oxidized materials, each of which, alone or in combination, may modulate cytokine-activated adhesion molecule gene expression. Fatty acid hydroperoxides such as linoleyl hydroperoxide (13-HPODE) are produced from free fatty acids by lipoxygenases and are an important component of oxidized LDL.

It has been proposed that a generation of oxidized lipids is formed by the action of the cell lipoxygenase system and that the oxidized lipids are subsequently transferred to LDL. There is thereafter a propagation reaction within the LDL in the medium catalyzed by transition metals and/or sulfhydryl compounds. Previous investigations have demonstrated that fatty acid modification of cultured endothelial cells can alter their susceptibility to oxidant injury. Supplementation of saturated or monounsaturated fatty acids to cultured endothelial cells reduces their susceptibility to oxidant injury, whereas supplementation with polyunsaturated fatty acids (PUFA) enhances susceptibility to oxidant injury.

Using reverse-phase HPLC analysis of native and saponified lipid extracts of LDL, it has been demonstrated that 13-HPODE is the predominant oxidized fatty acid in LDL oxidized by activated human monocytes. Chronic exposure to oxidized LDL provides an oxidative signal to vascular endothelial cells, possibly through a specific

fatty acid hydroperoxide, that selectively augments cytokine-induced VCAM-1 gene expression.

Through a mechanism that is not well defined, areas of vessel wall predisposed to atherosclerosis preferentially sequester circulating LDL. Through a poorly understood pathway, endothelial, smooth muscle, and/or inflammatory cells then convert LDL to ox-LDL. In contrast to LDL, which is taken up through the LDL receptor, monocytes avidly take up ox-LDL through a "scavenger" receptor whose expression, unlike the LDL receptor, is not inhibited as the content of intracellular lipid rises. Thus, monocytes continue to take up ox-LDL and become lipid-engorged macrophage-foam cells that form the fatty streak.

Given that cardiovascular disease is currently the leading cause of death in the United States, and ninety percent of cardiovascular disease is presently diagnosed as atherosclerosis, there is a strong need to identify new methods and pharmaceutical agents for its treatment. Important to this goal is the identification and manipulation of the specific oxidized biological compounds that act as selective regulators of the expression of mediators of the inflammatory process, and in particular, VCAM-1. A more general goal is to identify selective methods for suppressing the expression of redox sensitive genes or activating redox sensitive genes that are suppressed.

It is therefore an object of the present invention to provide a treatment for atherosclerosis and other cardiovascular and inflammatory diseases.

It is another object of the present invention to provide a method for the selective inhibition of VCAM-1.

It is still another object of the present invention to provide a method for the treatment of a human disease or disorder that is mediated by the expression or suppression of a redox sensitive gene.

It is another object of the present invention to provide pharmaceutical compositions for the treatment of atherosclerosis and other cardiovascular and inflammatory diseases.

Summary of the Invention

It has been discovered that polyunsaturated fatty acids ("PUFAs") and their hydroperoxides ("ox-PUFAs"), which are important components of oxidatively modified low density lipoprotein (LDL), induce the expression of VCAM-1, but not intracellular adhesion molecule-1 (ICAM-1) or E-selectin in human aortic endothelial cells, through a mechanism that is not mediated by cytokines or other noncytokine signals. This is a fundamental discovery of an important and previously unknown biological pathway in VCAM-1 mediated immune responses.

As nonlimiting examples, linoleic acid, linolenic acid, arachidonic acid, linoleyl hydroperoxide (13-HPODE) and arachidonic hydroperoxide (15-HPETE) induce cell-surface gene expression of VCAM-1 but not ICAM-1 or E-selectin. Saturated fatty acids (such as stearic acid) and monounsaturated fatty acids (such as oleic acid) do not induce the expression of VCAM-1, ICAM-1, or E-selectin.

The induction of VCAM-1 by PUFAs and their fatty acid hydroperoxides is suppressed by the antioxidant pyrrolidine dithiocarbamate (PDTC). This indicates that the induction is mediated by an

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oxidized signal molecule, and that the induction is prevented when the oxidation of the molecule is blocked (i.e., the oxidation does not occur), reversed (i.e., the signal molecule is reduced), or
5 when the redox modified signal is otherwise prevented from interacting with its regulatory target.

Cells that are chronically exposed to higher than normal levels of polyunsaturated fatty acids
10 or their oxidized counterparts can initiate an immune response that is not normal and which is out of proportion to the threat presented, leading to a diseased state. The oversensitization of vascular endothelial cells to PUFAS and ox-PUFAS can
15 accelerate the formation, for example, of atherosclerotic plaque.

Based on these discoveries, a method for the treatment of atherosclerosis, post-angioplasty restenosis, coronary artery diseases, angina, and
20 other cardiovascular diseases, as well as noncardiovascular inflammatory diseases that are mediated by VCAM-1, is provided that includes the removal, decrease in the concentration of, or prevention of the formation of oxidized
25 polyunsaturated fatty acids including but not limited to oxidized linoleic ($C_{18} \Delta^{9,12}$), linolenic ($C_{18} \Delta^{6,9,12}$), arachidonic ($C_{20} \Delta^{5,8,11,14}$) and eicosatrienoic ($C_{20} \Delta^{8,11,14}$) acids.

Nonlimiting examples of noncardiovascular
30 inflammatory diseases that are mediated by VCAM-1 include rheumatoid and osteoarthritis, asthma, dermatitis, and multiple sclerosis.

This method represents a significant advance in treating cardiovascular disease, in that it goes
35 beyond the current therapies designed simply to inhibit the progression of the disease, and when used appropriately, provides the possibility to

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medically "cure" atherosclerosis by preventing new lesions from developing and causing established lesions to regress.

In an alternative embodiment, a method is provided for suppressing the expression of a redox-sensitive gene or activating a gene that is suppressed through a redox-sensitive pathway, that includes administering an effective amount of a substance that prevents the oxidation of the oxidized signal, and typically, the oxidation of a polyunsaturated fatty acid. Representative redox-sensitive genes that are involved in the presentation of an immune response include, but are not limited to, those expressing cytokines involved in initiating the immune response (e.g., IL-1 β), chemoattractants that promote the migration of inflammatory cells to a point of injury (e.g., MCP-1), growth factors (e.g., IL-6 and the thrombin receptor), and adhesion molecules (e.g., VCAM-1 and E-selectin).

Screens for disorders mediated by VCAM-1 or a redox-sensitive gene are also provided that include the quantification of surrogate markers of the disease. In one embodiment, the level of oxidized polyunsaturated fatty acid, or other appropriate markers, in the tissue or blood, for example, of a host is evaluated as a means of assessing the "oxidative environment" of the host and the host's susceptibility to VCAM-1 or redox-sensitive gene mediated disease.

In another embodiment, the level of circulating or cell-surface VCAM-1 or other appropriate marker and the effect on that level of administration of an appropriate antioxidant is quantified.

In yet another assay, the sensitization of a host's vascular endothelial cells to polyunsaturated fatty acids or their oxidized

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counterparts is evaluated. This can be accomplished, for example, by challenging a host with a PUFA or ox-PUFA and comparing the resulting concentration of cell-surface or circulating VCAM-1 or other surrogate marker to a population norm.

In another embodiment, in vivo models of atherosclerosis or other heart or inflammatory diseases that are mediated by VCAM-1 can be provided by administering to a host animal an excessive amount of PUFA or oxidized polyunsaturated fatty acid to induce disease. These animals can be used in clinical research to further the understanding of these disorders.

In yet another embodiment of the invention, compounds can be assessed for their ability to treat disorders mediated by VCAM-1 on the basis of their ability to inhibit the oxidation of a polyunsaturated fatty acid, or the interaction of a PUFA or ox-PUFA with a protein target.

This can be accomplished by challenging a host, for example, a human or an animal such as a mouse, to a high level of PUFA or ox-PUFA and then determining the therapeutic efficacy of a test compound based on its ability to decrease circulating or cell surface VCAM-1 concentration. Alternatively, an in vitro screen can be used that is based on the ability of the test compound to prevent the oxidation of a PUFA, or the interaction of a PUFA or ox-PUFA with a protein target in the presence of an oxidizing substance such as a metal, for example, copper, or an enzyme such as a peroxidase, lipxygenase, cyclooxygenase, or cytochrome P450.

In another embodiment, vascular endothelial cells are exposed to TNF- α or other VCAM-1 inducing material for an appropriate time and then broken by any appropriate means, for example by

-10-

sonication or freeze-thaw. The cytosolic and membrane compartments are isolated. Radiolabeled PUFA is added to defined amounts of the compartments. The ability of the liquid to convert
5 PUFA to ox-PUFA in the presence or absence of a test compound is assayed. Intact cells can be used in place of the broken cell system.

Pyrrolidine dithiocarbamate (PDTC), orally delivered at 25-50mg/kg/day, dramatically inhibited
10 atherogenic fatty streak formation, arterial monocyte-macrophage inflammation, endothelial VCAM-1 expression and essentially normalized endothelium dependent relaxation function in diet induced hypercholesterolemic rabbits with serum cholesterol
15 levels over 1000 mg/dl. At the same doses, other putative therapeutic agents, such as the antioxidants probucol and vitamin E, had no effect on lesion formation in this model.

Endothelial dependent arterial relaxation is
20 restored in experimental atherosclerosis by administration of PDTC. In the diet induced hypercholesterolemic rabbit model, orally delivered (25-50mg/kg/day) PDTC restored endothelial dependent vasoreactivity. This was determined by
25 ring-contraction studies of excised aorta from control and test animals. In patients with atherosclerosis, this manifests itself as normalization of peripheral vascular reactivity in response to hyperemia as measured by non-invasive
30 Doppler flow studies. This is a standardized, commonly available and easily administered test that can be used to titrate functional drug levels to oral doses. The PDTC functions as an anti-ischemic therapy by rapidly normalizing the
35 pathological loss of endothelial derived arterial vasodilation characteristic of cardiovascular diseases and atherosclerosis. This improvement in

-11-

vascular blood flow is manifested as an improvement in symptom and ischemia-limited exercise function and provides a non-invasive assessment of vascular protection. Other clinical indications of

5 abnormalities in endothelial derived vasorelaxation include impotence.

The molecular regulator factory that controls VCAM-1 gene transcription is a novel transcription factor complex consisting of the p65 and p50

10 subunits of the NF-kB/Rel family cross-coupled to the c-fos and the c-jun subunits of the AP-1 family. By both structural and functional studies, it has been established that these AP-1 factors

15 play an important role in the regulation of the VCAM-1 promoter that likely are central to therapeutic regulation of VCAM-1 gene expression. This is the first demonstration of a functional role of this cross-coupled transcription complex in the regulation of an endogenous gene.

20 **Brief Description of the Figures**

Figure 1 is a graph of the cell-surface expression (O.D. 450 nm) of VCAM-1 as a function of hours in human aortic endothelial cells on exposure to the cytokine TNF- α (closed circle); linoleic

25 acid (closed triangle); and linoleyl hydroperoxide (13-HPODE, closed square); and in the absence of exposure to these substances (control, open square).

Figure 2 is a graph of the cell-surface expression (O.D. 450 nm) of VCAM-1 in human aortic

30 endothelial cells on exposure to linoleic acid (closed triangle) and linoleyl hydroperoxide (13-HPODE, closed square) as a function of the concentration of fatty acid (μ M).

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Figure 3 is a bar chart graph of the cell-surface expression (O.D. 450 nm) of VCAM-1, ICAM-1 and E-selectin in human aortic endothelial cells on exposure to the cytokine TNF- α , stearic acid, oleic acid, linoleic acid, linolenic acid, and arachidonic acid.

Figure 4 is a bar chart graph of the cell-surface expression (O.D. 450 nm) of VCAM-1 in human aortic endothelial cells on exposure to linoleic acid, 13-HPODE, arachidonic acid, and arachidonic acid hydroperoxide (15-HPETE), with (solid black) or without (hatched lines) the antioxidant pyrrolidine dithiocarbamate.

Figure 5 is an illustration of an autoradiogram indicating the acute induction of VCAM-1 mRNA by linoleic acid and 13-HPODE. HAEC were exposed or not to linoleic acid (7.5 μ M), 13-HPODE (7.5 μ M) or TNF- α (100 U/ml). Total RNA was isolated and 20 μ g was size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose, and hybridized to either 32 P-labeled human A) VCAM-1 specific or B) β -actin specific cDNA. After washing, the filters were exposed to X-ray film at -70°C with one intensifying screen for 24 hours. Identification of lanes: 1) control; 2) linoleic acid (acute, 8-hour exposure); 3) linoleic acid (48-hour exposure); 4) 13-HPODE (acute, 8-hour exposure); and 5) TNF- α (100 U/ml, 4-hour exposure).

Figure 6 is an illustration of an autoradiogram that indicates that induction of VCAM-1 mRNA by polyunsaturated fatty acids is independent of cellular protein synthesis. HAEC were exposed to either linoleic (7.5 μ M) or arachidonic (7.5 μ M) acid in the presence or absence of cycloheximide (10 μ g/ml) for a 4-hour period, and then treated as described in Figure 5.

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Figure 7 is an illustration of an autoradiogram that indicates that linoleic acid induces transcriptional activation of the VCAM-1 promoter by a redox-sensitive NF-kB like factor. HAEC were split at the ratio to give approximately 60% confluence in 100-mm tissue culture plates. HAEC were transfected with either 30 μ g of p288 VCAMCAT, p85 VCAMCAT, or pSV₂CAT plasmid by the calcium phosphate coprecipitation technique using standard techniques. After a 24-hour recovery period, HAEC were pretreated or not with 50 μ M PDTC and after 30 minutes exposed to linoleic acid (7.5 μ M) or TNF- α (100 U/ml) directly added to the plates. After 18 hours, cell extracts were prepared by rapid freeze-thaw in 0.25 M Tris, pH 8.0. The protein of each cell extract was assayed for chloramphenicol acetyl transferase (CAT) activity, as previously described [Ausubel, 1989] (Ac, acetylated; N, nonacetylated chloramphenicol).

Figure 8 is an illustration of an acrylamide gel slab that indicates that polyunsaturated fatty acids activate NF-kB-like DNA binding activities that are blocked by the antioxidant PDTC. Confluent HAEC in media containing 4% FBS (as described in Figure 1) were pretreated or not with PDTC (50 μ M) for thirty minutes and then exposed for three hours to linoleic acid (7.5 μ M, oleic acid (7.5 μ M), or TNF α (100 U/ml), respectively. Five micrograms of nuclear extract was incubated with a double-stranded ³²P-labeled wtVCAM, size fractionated on 4% native acrylamide gels, and exposed to autoradiography film at -70°C for 18 hours. Two bands A and C, representing NF-kB like binding activity are designated. A weak band B was observed in control (untreated) cells.

Figures 9A and 9B are bar chart graphs of the relative thiobarbituric acid reactive substances

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(O.D. 532 nm) of arachidonic acid and 15-HPETE in the presence or absence of PDTC. The thiobarbituric acid reactivity assay (TBARS) measures the oxidation ability of a material in a cell-free, media-free environment.

Figure 10 is an illustration of an autoradiogram of mRNA, obtained as described below, hybridized to either 32P-labeled human VCAM-1 specific cDNA (Panel A), E-selectin (ELAM-1) specific cDNA (Panel B), or ICAM-1 specific cDNA (Panel C). Following pre-treatment for 30 minutes with 50 μ M of sodium pyrrolidine dithiocarbamate (PDTC), HUVE (human umbilical vein) cells were exposed to IL-1b (10 U/ml) in the continuous presence of 50 μ M PDTC. Parallel controls were performed identically except in the absence of PDTC. At the indicated times, total RNA was isolated and 20 μ g of material size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose, hybridized as described above, and visualized by autoradiography. Lane 1-0 hour; Lanes 2,4,6,8 - OL-1 alone for 2, 4, 8 and 24 hours, respectively; Lanes 3,5,7,9 - IL-1 with PDTC for 2,4,8 and 24 hours, respectively.

Figure 11 is an illustration of an autoradiogram of mRNA, obtained as described below, hybridized to either 32P-labeled human VCAM-1 specific (Panel A), E-selectin (ELAM-1) specific cDNA (Panel B), or ICAM-1 specific cDNA (Panel C). HUVE cells were pretreated with the indicated concentrations of PDTC, and then exposed to IL-1b in the presence of PDTC for four hours and assayed for VCAM-1 mRNA accumulation by Northern filter hybridization analysis. Lane 1 - control, lane 2 - IL-1 (10u/ml), lane 3 - IL-1b + PDTC (0.05 μ M), lane 4 - IL-1 LB + PDTC (0.5 μ M), lane 5 - IL-1b + PDTC (5.0 μ M), lane 6 - IL=1b + PDTC (50.0 μ M), lane 7

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- IL-1b + PDTC (100 μ M).

Figure 12 is an illustration of an autoradiogram of mRNA, obtained as described below, hybridized to either ³²P-labeled human VCAM-1 specific cDNA

5 (Panel A), E-selectin (ELAM-1) specific cDNA (Panel B), or ICAM-1 specific cDNA (Panel C). HUVE cells were pretreated as described in Figure 9 with 50 μ M PDTC, exposed for four hours to the agents indicated below, and assayed for VCAM-1 (Panel A)
10 and ICAM-1 (Panel B) mRNA accumulation. Lane 1 - TNFa (100U/ml), lane 2 - TNFa + PDTC, lane 3 - lipopolysaccharide (LPS) (100ng/ml), lane 4 - LPS + PDTC, lane 5 - poly(I:C) (100mg/ml), lane 6 - poly(I:C) + PDTC.

15 Figure 13 is a graph of relative cell surface expression of VCAM-1 and ICAM-1 in the presence (dark bars) or absence (white bars) of PDTC and in the presence of multiple types of inducing stimuli. Confluent HUVECs were pretreated or not pretreated
20 (CTL only) for 30 minutes with 50 μ M PDTC, and then exposed for the indicated times to the indicated agents in the presence or absence (CTL only) of PDTC. Cell surface expression was determined by primary binding with VCAM-1 specific (4B9) and
25 ICAM-1 specific (84H10) mouse monoclonal antibodies followed by secondary binding with a horse-radish peroxidase tagged goat anti-mouse (IgG). Quantitation was performed by determination of calorimetric conversion at 450 nm of TMB. Figure
30 13 indicates that multiple regulatory signals induce VCAM-1 but not ICAM-1 through a common, dithiocarbamate-sensitive pathway in human vascular endothelial cells.

Figure 14 is a graph of the relative VCAM-1 cell
35 surface expression (O.D. 595 nm) in human umbilical vein endothelial cells, activated by TNFa, versus concentration of various antioxidants. (PDTC is

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sodium N-pyrrolidine dithiocarbamate; DETC is sodium N,N-diethyl-N-carbodithiolate, also referred to as sodium diethyldithiocarbamate; NAC is N-acetyl cysteine; and DF is desferroximine).

5 Figure 15 is a graph of the relative VCAM-1 cell surface expression (O.D. 595 nm) in human umbilical vein endothelial cells, activated by TNF- α , in the presence of the specified amount of antioxidant. (PDTC is sodium N-pyrrolidine dithiocarbamate; 10 DIDTC is sodium N,N-diethyl-N-carbodithioate; SarDTC is sodium N-methyl-N-carboxymethyl-N-carbodithioate; IDADTC is trisodium N,N-di(carboxymethyl)-N-carbodithioate; MGDTC is sodium N-methyl-D-glucamine-N-carbodithioate; MeOBGDTC is 15 sodium N-(4-methoxybenzyl)-D-glucamine-N-carbodithioate; DEDTC is sodium N,N-diethyl-N-carbodithioate; Di-PDTC is sodium N,N-diisopropyl-N-carbodithioate; NAC is N-acetyl cysteine.)

20 Figure 16 is a graph of the percentage of Molt-4 cells binding to HUVE cells either unstimulated or stimulated with TNFa (100 U/ml) for six hours in the presence or absence of PDTC.

25 Figure 17 is an illustration of the chemical structures of the following active dithiocarbamates: sodium pyrrolidine-N-carbodithioate, sodium N-methyl-N-carboxymethyl-N-carbodithioate, trisodium N,N-di(carboxymethyl)-N-carbodithioate, sodium N-methyl-D-glucamine-N-carbodithioate, sodium N,N-diethyl-N-carbodithioate (sodium diethyldithiocarbamate), and sodium N,N- 30 diisopropyl-N-carbodithioate.

35 Figure 18 is a bar chart graph of the effect of PDTC on the formation of fluorescent adducts of BSA and 13-HPODE, as measured in fluorescent units versus micromolar concentration of PDTC. One micromolar of 13-HPODE was incubated with 200 micrograms of BSA in the presence of PDTC for six

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days. Fluorescence was measured at 430-460 nm with excitation at 330-360 nm.

Figure 19 is a graph of the effect of PTDC on the formation of fluorescent adducts of BSA and ox-
 5 PUFA as a function of wavelength (nm) and concentration of PDTC. As the concentration of PDTC increases, the quantity of fluorescent adducts decrease.

Figure 20 is a graph of the effect of PDTC on
 10 the oxidation of LDL by horseradish peroxidase (HRP), as measured by the increase in O.D. (234 nm) versus time (minutes) for varying concentrations of PDTC. It is observed that after an incubation period, PDTC inhibits the oxidation of LDL by HRP
 15 in a manner that is concentration dependent.

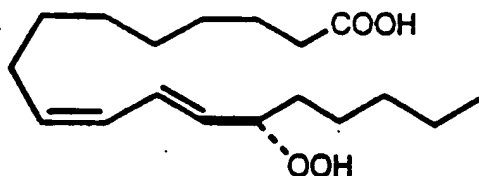
Figure 21 is a chart of the effect of PDTC on the cytokine-induced formation of ox-PUFA in human aortic endothelial cells. As indicated, both TNF- α and IL-1B causes the oxidation of linoleic acid to
 20 ox-linoleic acid. The oxidation is significantly prevented by PDTC.

Detailed Description of the Invention

I. Definitions

As used herein, the term polyunsaturated fatty
 25 acid (also referred to herein as a "PUFA") refers to a fatty acid (typically C_8 to C_{24}) that has at least two alkenyl bonds, and includes but is not limited to linoleic ($C_{18} \Delta^{9,12}$), linolenic ($C_{18} \Delta^{6,9,12}$), arachidonic ($C_{20} \Delta^{5,8,11,14}$) and eicosatrienoic
 30 ($C_{20} \Delta^{8,11,14}$) acids.

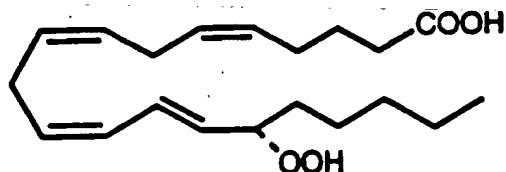
The term oxidized polyunsaturated fatty acid refers to an unsaturated fatty acid in which at least one of the alkenyl bonds has been converted to a hydroperoxide. Nonlimiting examples are:



13-HPODE

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15-HPETE



The term alkyl, as used herein, unless otherwise specified, refers to a saturated straight, branched, or cyclic (in the case of C_3 or greater) hydrocarbon of C_1 to C_{10} (or lower alkyl, i.e., C_1 to C_3), which specifically includes methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, cyclopentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, cyclohexylmethyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl. The alkyl group can be optionally substituted on any of the carbons with one or more moieties selected from the group consisting of hydroxyl, amino, or mono- or disubstituted amino, wherein the substituent group is independently alkyl, aryl, alkaryl or aralkyl; aryl, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, et al., "Protective Groups in Organic Synthesis," John Wiley and Sons, Second Edition, 1991.

The term alkenyl, as referred to herein, and unless otherwise specified, refers to a straight, branched, or cyclic hydrocarbon of C_2 to C_{10} with at least one double bond.

The term alkynyl, as referred to herein, and unless otherwise specified, refers to a C_2 to C_{10} straight or branched hydrocarbon with at least one triple bond.

The term aralkyl refers to an aryl group with at least one alkyl substituent.

The term alkaryl refers to an alkyl group that has at least one aryl substituent.

The term halo (alkyl, alkenyl, or alkynyl) refers to an alkyl, alkenyl, or alkynyl group in which at least one of the hydrogens in the group has been replaced with a halogen atom.

The term aryl, as used herein, and unless otherwise specified, refers to phenyl, biphenyl, or naphthyl, and preferably phenyl. The aryl group can be optionally substituted with one or more moieties selected from the group consisting of alkyl, hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, CO_2H , or its pharmaceutically acceptable salt, $\text{CO}_2(\text{alkyl, aryl, alkaryl or aralkyl})$, or glucamine, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, et al., "Protective Groups in Organic Synthesis," John Wiley and Sons, Second Edition, 1991.

The term alkoxy, as used herein, and unless otherwise specified, refers to a moiety of the structure $-\text{O}-\text{alkyl}$.

The term acyl, as used herein, refers to a group of the formula $\text{C}(\text{O})\text{R}'$, wherein R' is an alkyl, aryl, alkaryl or aralkyl group.

The term heteroaryl or heteroaromatic, as used herein, refers to an aromatic moiety that includes at least one sulfur, oxygen, or nitrogen in the aromatic ring. Nonlimiting examples are phenazine, phenothiazine, furyl, pyridyl, pyrimidyl, thienyl, isothiazolyl, imidazolyl, tetrazolyl, pyrazinyl, benzofuranyl, benzothiophenyl, quinolyl, isoquinolyl, benzothienyl, isobenzofuryl, pyrazolyl, indolyl, isoindolyl, benzimidazolyl, purinyl, morpholinyl, carbozolyl, oxazolyl,

thiazolyl, isothiazolyl, 1,2,4-thiadiazolyl, isooxazolyl, pyrrolyl, pyrazolyl, quinazolinyl, pyridazinyl, pyrazinyl, cinnolinyl, phthalazinyl, quinoxalinyl, xanthinyl, hypoxanthinyl, pteridinyl, 5 5-azacytidinyl, 5-azauracilyl, triazolopyridinyl, imidazolopyridinyl, pyrrolopyrimidinyl, pyrazolopyrimidinyl, adenine, N⁶-alkylpurines, N⁶-benzylpurine, N⁶-halopurine, N⁶-vinylpurine, N⁶-acetylenic purine, N⁶-acyl purine, 10 N⁶-hydroxyalkyl purine, N⁶-thioalkyl purine, thymine, cytosine, 6-azapyrimidine, 2-mercaptopyrimidine, uracil, N⁵-alkylpyrimidines, N⁵-benzylpyrimidines, N⁵-halopyrimidines, N⁵-vinylpyrimidine, N⁵-acetylenic pyrimidine, N⁵-acyl 15 pyrimidine, N⁵-hydroxyalkyl purine, and N⁶-thioalkyl purine, and isoxazolyl. The heteroaromatic group can be optionally substituted as described above for aryl. The heteroaromatic can be partially or totally hydrogenated as desired. As a nonlimiting 20 example, dihydropyridine can be used in place of pyridine. Functional oxygen and nitrogen groups on the heterocyclic base can be protected as necessary or desired during the reaction sequence. Suitable protecting groups are well known to those 25 skilled in the art, and include trimethylsilyl, dimethylhexylsilyl, t-butyldimethylsilyl, and t-butyldiphenylsilyl, tritylmethyl, alkyl groups, acyl groups such as acetyl and propionyl, methylsulfonyl, and p-toluylsulfonyl.

30 The term hydroxyalkyl, as used herein, refers to a C₁ to C₆ alkyl group in which at least one of the hydrogens attached to any of the carbon atoms is replaced with a hydroxy group.

The term thiol antioxidant refers to a sulfur 35 containing compound that retards oxidation.

The term pharmaceutically acceptable derivative refers to a derivative of the active compound that

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upon administration to the recipient, is capable of providing directly or indirectly, the parent compound, or that exhibits activity itself.

The term "pharmaceutically acceptable cation" refers to an organic or inorganic moiety that carries a positive charge and that can be administered in association with a pharmaceutical agent, for example, as a countercation in a salt. Pharmaceutically acceptable cations are known to those of skill in the art, and include but are not limited to sodium, potassium, and quaternary amine.

The term "physiologically cleavable leaving group" refers to a moiety that can be cleaved in vivo from the molecule to which it is attached, and includes but is not limited to an organic or inorganic anion, a pharmaceutically acceptable cation, acyl (including but not limited to (alkyl)C(O), including acetyl, propionyl, and butyryl), alkyl, phosphate, sulfate and sulfonate.

The term "enantiomerically enriched composition or compound" refers to a composition or compound that includes at least 95%, and preferably at least 97, 98, 99, or 100% by weight of a single enantiomer of the compound.

The term amino acid includes synthetic and naturally occurring amino acids, including but not limited to, for example, alanyl, valinyl, leucinyl, isoleucinyl, prolinyl, phenylalaninyl, tryptophanyl, methioninyl, glycyl, serinyl, threoninyl, cysteinyl, tyrosinyl, asparaginyl, glutaminyl, aspartoyl, glutaoyl, lysinyl, argininyl, and histidinyl.

A "linking moiety" as used herein, is any divalent group that links two chemical residues, including but not limited to alkyl, alkenyl, alkynyl, aryl, polyalkyleneoxy (for example $-[(CH_2)_nO-]_n-$), $-C_{1-6}alkoxy-C_{1-10}alkyl-$,

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-C₁₋₆alkylthio-C₁₋₁₀ alkyl-, -NR³-, and -(CHOH)_nCH₂OH,
wherein n is independently 0, 1, 2, 3, 4, 5, or 6.

II. Identification of Oxidized and Unoxidized
Polyunsaturated Fatty Acids as Direct
Mediators of VCAM-1 Expression

To establish whether a PUFA or oxidized PUFA acts as a direct immunomodulator of endothelial cell gene expression, early passaged human aortic endothelial cells (HAEC) were cultured for eight hours in media and serum and exposed to saturated (stearic), monounsaturated (oleic), and polyunsaturated (linoleic and arachidonic) fatty acids; as well as with the fatty acid hydroperoxides of linoleic (13-HPODE) or arachidonic (15-HPETE) acids. HAEC were also alternatively exposed to the cytokine tumor necrosis factor- α .

HAEC were exposed to linoleic acid or 13-HPODE for varying times up to 48 hours and then assayed for cell surface VCAM-1 expression by ELISA assay. The results were compared to HAEC exposed to the cytokine TNF- α (100 U/ml) for the same time periods. VCAM-1 expression in HAEC incubated with either linoleic acid or 13-HPODE is transiently induced. The expression peaks at approximately 8-9 hours with significant expression at 24 hours and then decreases by 48 hours. The kinetics of VCAM-1 induction by both linoleic acid and 13-HPODE mirror that of TNF- α , and thus the mechanisms by which polyunsaturated fatty acids induce VCAM-1 thus appear to be similar to that of TNF- α .

Dose-response studies of linoleic acid and 13-HPODE on VCAM-1 gene expression at 8 hours were also conducted. It was observed that 7.5 μ M is the lowest peak dose by which linoleic acid and

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13-HPODE induces significant VCAM-1 gene expression.

It was then explored whether short term incubation of endothelial cells with
5 polyunsaturated fatty acids induces ICAM-1 and E-selectin expression as well. It was determined that the polyunsaturated fatty acids linoleic and arachidonic acids induced cell-surface gene
10 expression to approximately 59% of TNF-induced gene expression of VCAM-1. Strikingly, neither ICAM-1 nor E-selectin were induced by these fatty acids. Conversely, the saturated fatty acid stearic acid and the monounsaturated fatty acid oleic acid did not induce the expression of VCAM-1, ICAM-1, or
15 E-selectin. VCAM-1 gene expression was also observed by incubation of HAEC with the oxidized metabolites of linoleic acid (13-HPODE) and arachidonic acid (15-HPETE).

To investigate whether oxidative stress in
20 endothelial cells provided by polyunsaturated fatty acids and their oxidized metabolites induces VCAM-1 through a redox-sensitive mechanism, HAEC were pretreated with the antioxidant pyrrolidine dithiocarbamate (PDTC, 50 μ M) for 30 minutes and
25 then the cells were independently incubated with linoleic acid, arachidonic acid, 13-HPODE, and 15-HPETE (all 7.5 μ M) for 8 hours. It was determined that PDTC suppressed the gene expression of VCAM-1 induced by the polyunsaturated fatty
30 acids and their oxidized counterparts. This indicates that the induction is mediated by a oxidized signal molecule, and that the induction is prevented when the oxidation of the molecule is blocked (i.e., the oxidation does not occur),
35 reversed (i.e., the signal molecule is reduced), or its interaction with a target protein prevented, perhaps through a redox complex.

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To determine whether the selective induction of VCAM-1 by PUFAs and their oxidized metabolites is observed at the mRNA level, HAEC were incubated with linoleic acid or 13-HPODE. Linoleic acid and 13-HPODE induced VCAM-1 mRNA accumulation that was similar to levels induced by TNF- α . In contrast, there was no induction of ICAM-1 or E-selectin gene expression at the mRNA level in HAEC incubated with linoleic acid or 13-HPODE. The findings mimic those found at the cell-surface level. These results indicate that pretranslational regulatory mechanisms mediate induction of VCAM-1 gene expression by polyunsaturated fatty acids and their oxidative metabolites.

It was also desired to determine whether polyunsaturated fatty acids work as a primary signal or operate through a regulatory protein involving the cytokine IL-4 in inducing VCAM-1 gene expression. To investigate whether newly synthesized proteins such as IL-4 are involved in the synthesis and gene expression of VCAM-1 induced by PUFAs such as linoleic acid, HAEC were incubated with 13-HPODE (7.5 μ M) and exposed to the protein synthesis inhibitor, cycloheximide. There was no inhibition of mRNA accumulation of VCAM-1 by cycloheximide in HAEC incubated with 13-HPODE. The production of IL-4 by HAEC incubated with linoleic or arachidonic acids and their oxidative metabolites, as determined by ELISA was also measured. There was no increase in IL-4 output by HAEC incubated with these PUFAs or their oxidized metabolites.

Previous investigations have demonstrated through deletion and heterologous promoter studies that cytokines and non-cytokines activate VCAM-1 gene expression in endothelial cells at least in part transcriptionally through two NF-kB-like DNA

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binding elements. It has also been demonstrated that PDTC inhibits VCAM-1 gene expression through a redox-sensitive NF-kB like factor. To determine whether polyunsaturated fatty acids induce transcriptional activation of the human VCAM-1 promoter via a similar mechanism, the chimeric reporter gene p288 VCAM-CAT, containing coordinates -288 to +22 of the human VCAM-1 promoter, was transiently transfected into HAEC. The addition of linoleic acid (7.5 μ M) induced VCAM-1 promoter. The addition of linoleic acid (7.5 μ M) induced VCAM-I promoter activity that was over two fold that of the control and approximately 60% of the maximum signal induced by TNF- α . Similar results were obtained with the minimal cytokine-inducible promoter of the VCAM-1 gene (p85 VCAM-CAT), containing the -77 and -63 bp NF-kB-like sites. Neither linoleic acid nor TNF- α had any effect on activity using a constitutively expressed pSV₂ CAT construct. PDTC inhibited the transcriptional activation of both VCAM-1 promoter constructs induced by linoleic acid. The data indicate that analogous to TNF- α , polyunsaturated fatty acids such as linoleic acid induce the transcriptional activation of VCAM-1 through an NF-kB-like redox-sensitive mechanism.

To determine whether polyunsaturated fatty acids and their oxidative metabolites regulate VCAM-1 promoter activity through an NF-kB-like transcriptional regulatory factor, nuclear extracts from HAEC were assayed for DNA binding activity to a double-stranded oligonucleotide containing the VCAM-1 NF-kB-like promoter elements located at positions -77 and -63. As shown in Figure 7, two bands A and C, representing NF-kB-like activity were induced in response to a three hour exposure to linoleic acid (7.5 μ M). Similar findings were

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observed on exposure to the cytokine TNF- α (100 U/ml). A weak band B was observed in control (untreated) cells. No induction of NF-kB-like binding was observed with the monounsaturated fatty acid oleic acid. Pretreatment of the cells for thirty minutes with PDTC inhibited the A and C complex DNA binding activity after linoleic acid activation. These findings are similar to previously reported findings that PDTC blocks the activation of VCAM-1 gene expression in HUVEC by inhibiting the activation of these NF KB-like DNA binding proteins.

Example 1 Effect of Oxidized and Unoxidized Polyunsaturated Fatty Acids on the Kinetics of the Activation of VCAM-1 Gene Expression

Human aortic endothelial cells (HAEC) were plated in 96 well plates and incubated with linoleic acid (7.5 μ M), 13-HPODE (7.5 μ M), or TNF- α (100 U/ml) at five different time points up to 48 hours. HAEC, obtained from Clonetics (Boston, MA), were cultured in Medium 199 supplemented with 20% fetal bovine serum (FBS), 16 U/ml heparin, 10 U/ml epidermal growth factor, 50 μ g/ml endothelial cell growth supplement, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. One day before the experiment, cells were placed in a medium containing 4% FBS. Confluent HAEC were incubated for up to 48 hours with TNF- α (100 U/ml), or stearic, oleic, linoleic, linolenic, or arachidonic acids (7.5 μ M). Similar studies were performed with differing doses of linoleic acid or 13-HPODE for an 8 hour period (1-60 μ M) (Figure 2). Quantitation was performed by determination of colorimetric conversion at 450 nm of TMB. Studies were performed in triplicate (n=4

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for each experimental value). *-value differs (p<0.05) from Control.

As shown in Figure 1, both linoleic acid and 13-HPODE induced the expression of VCAM-1. At ten hours after exposure, the amount of cell surface VCAM-1 induced by linoleic acid and 13-HPODE was greater than half that induced by the cytokine TNF- α .

As shown in Figure 2, the induction of VCAM-1 by linoleic acid and 13-HPODE is concentration sensitive. At a concentration of between 2 and 10 μ M of these compounds, there is a sharp increase in the amount of induced cell surface VCAM-1, which then remains approximately constant up to a concentration of at least 100 μ M. It should be observed that the PUFA concentration indicated in Figure 2 is in addition to that found endogenously in HAEC.

Example 2 Polyunsaturated Fatty Acids Induce Gene Expression of VCAM-1 but not ICAM-1 or E-selectin.

The cell surface expression of VCAM-1, ICAM-1, and E-selectin was measured in HAEC by ELISA. HAEC, obtained from Clonetics (California), were cultured in Medium 199 supplemented with 20% fetal bovine serum (FBS), 16 U/ml heparin, 10 U/ml epidermal growth factor, 50 μ g/ml endothelial cell growth supplement, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. One day before the experiment, cells were placed in a medium containing 4% FBS. Confluent HAEC were incubated or not for 8 hours with TNF- α (100 U/ml), or stearic, oleic, linoleic, linolenic, or arachidonic acids (7.5 μ M). Cell-surface expression of A) VCAM-1, B) ICAM-1, and C) E-selectin was determined by primary binding with VCAM-1 specific, ICAM-1 specific, and E-selectin

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specific mouse antibodies followed by secondary binding with a horseradish peroxidase-tagged goat anti-mouse (IgG). Quantitation was performed by determination of colorimetric conversion at 450 nm of TMB. Studies were performed in triplicate (n=4 for each experimental value). *-value differs (p<0.05) from Control.

As shown in Figure 3, linoleic acid, linolenic acid, and arachidonic acid significantly induced the expression of VCAM-1, but did not induce the cell-surface expression of ICAM-1 or E-selectin. Neither stearic acid nor oleic acid induced the expression of VCAM-1, ICAM-1, or E-selectin. TNF- α strongly induced the expression of all three cell-surface molecules.

Example 3 The Antioxidant PDTC Suppresses VCAM-1 Induction by Polyunsaturated Fatty Acids and their Oxidative Metabolites.

Confluent HAEC were pretreated in the presence or absence of PDTC (sodium pyrrolidine dithiocarbamate, 50 μ M) for thirty minutes. The cells were then incubated for eight hours with TNF- α (100 U/ml), linoleic or arachidonic acid (7.5 μ M), or the fatty acid hydroperoxides 13-HPODE (7.5 μ M) or 15-HPETE (7.5 μ M). The cell surface expression of VCAM-1 was measured in HAEC by ELISA, as described in Example 1. Studies were performed in triplicate (n = 4 for each experimental value). *-value differs (p<0.05) from control.

As indicated in Figure 4, PDTC suppresses the induction of VCAM-1 by linoleic acid, 13-HPODE, arachidonic acid and 15-HPETE.

Example 4 Acute Induction of VCAM-1 mRNA by Linoleic Acid and 13-HPODE.

HAEC were exposed to linoleic acid (7.5 μ M) or 13-HPODE (7.5 μ M). Total RNA was isolated and 20

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μ g size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose, and hybridized to either 32 P-labeled human A) VCAM-1 specific or B) β -actin specific cDNA and visualized by autoradiography. After washes, filters were exposed to X-ray film at -70°C with one intensifying screen for 24 hours. Identification of lanes: 1) control; 2) linoleic acid (acute, 8-hour exposure); 3) linoleic (48-hour exposure); 4) 13-HPODE (acute, 8-hour exposure); and 5) $\text{TNF-}\alpha$ (100 U/ml, 4-hour exposure).

As shown in Figure 5, both linoleic acid and 13-HPODE induce the production of mRNA for VCAM-1 in eight hours. After 48 hours, linoleic acid no longer induces VCAM-1 mRNA.

Example 5 Induction of VCAM-1 mRNA by PUFAs is Independent of Cellular Protein Synthesis.

HAEC were exposed to either linoleic or arachidonic acid ($7.5 \mu\text{M}$) in the presence or absence of cycloheximide ($10 \mu\text{g/ml}$) for a 4-hour period. Total RNA was isolated and $20 \mu\text{g}$ was size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose, and hybridized to A) ^{32}P -labeled human VCAM-1 or B) β -actin specific cDNA and then visualized by autoradiography. After washes, filters were exposed to X-ray film at -70°C with one intensifying screen for 24 hours.

As indicated in Figure 6, the induction of VCAM-1 by linoleic and arachidonic acids are independent of cellular protein synthesis.

-30-

Example 6 **Linoleic acid induces transcriptional activation of the VCAM-1 promoter by a redox-sensitive NF- κ B like factor.**

HAEC were split at the ratio to give
5 approximately 60% confluence in 100-mm tissue culture plates. HAEC were transfected with either 30 μ g of p288 VCAMCAT, p85 VCAMCAT, or pSV₂CAT plasmid by the calcium phosphate coprecipitation technique using standard techniques. After a
10 24-hour recovery period, HAEC were pretreated with 50 μ M PDTC and after 30 minutes exposed to linoleic acid (7.5 μ M) or TNF- α (100 U/ml) directly added to the plates. After 18 hours, cell extracts were prepared by rapid freeze-thaw in 0.25 M Tris, pH
15 8.0. Protein of each cell extract was assayed for chloramphenicol acetyl transferase (CAT) activity (Ac, acetylated; N, nonacetylated chloramphenicol).

Figure 7 illustrates the results of this experiment. Linoleic acid induces transcriptional
20 activation of the VCAM-1 promoter by a redox-sensitive NF- κ B like factor. These results are similar to those observed by the activation of VCAM-1 promoter by cytokines such as TNF- α . This suggests that PUFAs act through an oxidized
25 intermediate that also mediates the cytokine activation of VCAM-1.

Example 7 **Polyunsaturated Fatty Acids Activate NF- κ B-like DNA Binding Activities that are Blocked by the Antioxidant PDTC.**

30 Confluent HAEC in media containing 4% FBS (as described in Example 1) were pretreated with PDTC (50 μ M) for 30 minutes and then exposed for 3 hours to linoleic acid or oleic acid (7.5 μ M), or TNF- α (100 U/ml). Five micrograms of nuclear extract was
35 incubated with a double-stranded ³²P-labeled wtVCAM, size fractionated on 4% native acrylamide gels, and exposed to autoradiography film at -70°C for 18

-31-

hours. Two bands A and C, representing NF-kB like binding activity are designated. A weak band B was observed in control (untreated) cells.

Figure 8 illustrates that linoleic acid induces NF-kB binding activity to VCAM-1 promotor in a redox-sensitive manner. This is analogous to cytokine TNF- α and suggests a similar mechanism of action. TNF- α probably induces VCAM-1 through a mechanism that is mediated by an ox-PUFA.

10 **Example 8 Oxidation in a cell-free, media-free setup, by both unoxidized and oxidized (15-HPETE) arachidonic acid**

Figures 9A and 9B are bar chart graphs of the relative thiobarbituric acid reactive substances (O.D. 532 nm) of arachidonic acid and 15-HPETE in the presence or absence of PDTC. The thiobarbituric acid reactivity assay (TBARS) measures the oxidation ability of a material in a cell-free, media-free environment. As indicated in the Figures, both arachidonic acid and 15-HPETE showed significant TBARS activity that was inhibited by PDTC.

III. **Method for the Treatment of VCAM-1 Mediated Disorders**

25 The discovery that polyunsaturated fatty acids and their oxidized metabolites are selective, redox-sensitive immunomodulators provides a basis for the therapy of disorders that are mediated by VCAM-1 or by redox-sensitive genes.

30 A method for the treatment of atherosclerosis, post-angioplasty restenosis, coronary artery diseases, angina, and other cardiovascular diseases, as well as noncardiovascular inflammatory diseases that are mediated by VCAM-1 is provided
35 that includes the removal, decrease in the

concentration of, or prevention of the formation of oxidized polyunsaturated fatty acids, including but not limited to oxidized linoleic, linolenic, and arachidonic acids. In an alternative embodiment, a method for the treatment of these diseases is provided that includes the prevention of the interaction of a PUFA or ox-PUFA with a protein or peptide that mediates VCAM-1 expression.

Inhibition of the expression of VCAM-1 can be accomplished in a number of ways, including through the administration of an antioxidant that prevent the oxidation of a polyunsaturated fatty acid, by in vivo modification of the metabolism of PUFAs into ox-PUFAs, as described in more detail below.

1. Administration of Antioxidants

Any compound that reduces an ox-PUFA or which inhibits the oxidation of PUFA, and which is relatively nontoxic and bioavailable or which can be modified to render it bioavailable, can be used in this therapy. One of ordinary skill in the art can easily determine whether a compound reduces an ox-PUFA or inhibits the oxidation of PUFA using standard techniques.

Dithiocarboxylate Antioxidants

It has been discovered that dithiocarboxylates are useful in the treatment of atherosclerosis and other cardiovascular and inflammatory diseases. Dithiocarboxylates, including dithiocarbamates, can be used to block the ability of cells, including endothelial cells, to express VCAM-1 or to suppress the expression of a redox-sensitive gene or activate a gene that is suppressed through a redox-sensitive pathway.

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At least one of the compounds, pyrrolidine dithiocarbamate (PDTC), inhibits VCAM-1 gene expression at a concentration of less than 1.0 micromolar. This compounds also exhibits
5 preferential toxicity to proliferating or abnormally dividing vascular smooth muscle cells. Another dithiocarbamate, sodium N-methyl-N-carboxymethyl-N-carbodithioate, also inhibits the expression of VCAM-1, without significant effect on
10 ICAM-1, but does not exhibit preferential toxicity to abnormally dividing vascular smooth muscle cells. Another dithiocarbamate, sodium N-methyl-N-carboxymethyl-N-carbodithioate, also inhibits the expression of VCAM-1, without significant effect on
15 ICAM-1, but does not exhibit preferential toxicity to abnormally dividing vascular smooth muscle cells.

It has been discovered that pyrrolidine dithiocarbamate does not significantly block ELAM-1
20 or ICAM-1 expression, and therefore treatment with this compound does not adversely affect aspects of the inflammatory response mediated by ELAM-1 or ICAM-1. Thus, a generalized immunosuppression is avoided. This may avoid systemic complications
25 from generalized inhibition of adhesion molecules in the many other cell types known to express them. Other pharmaceutically acceptable salts of PDTC are also effective agents for the treatment of cardiovascular and inflammatory disorders.

30 Dithiocarbamates are transition metal chelators clinically used for heavy metal intoxication. Baselt, R.C., F.W.J. Sunderman, et al. (1977), "Comparisons of antidotal efficacy of sodium diethyldithiocarbamate, D-penicillamine and
35 triethylenetetramine upon acute toxicity of nickel carbonyl in rats." Res Commun Chem Pathol Pharmacol 18(4): 677-88; Menne, T. and K. Kaaber

-34-

- (1978), "Treatment of pompholyx due to nickel allergy with chelating agents." Contact Dermatitis 4(5): 289-90; Sunderman, F.W. (1978), "Clinical response to therapeutic agents in poisoning from mercury vapor" Ann Clin Lab Sci 8(4): 259-69; Sunderman, F.W. (1979), "Efficacy of sodium diethyldithiocarbamate (dithiocarb) in acute nickel carbonyl poisoning." Ann Clin Lab Sci 9(1): 1-10; Gale, G.R., A.B. Smith, et al. (1981), "Diethyldithiocarbamate in treatment of acute cadmium poisoning." Ann Clin Lab Sci 11(6): 476-83; Jones, M.M. and M.G. Cherian (1990), "The search for chelate antagonists for chronic cadmium intoxication." Toxicology 62(1): 1-25; Jones, S.G., M.A. Basinger, et al. (1982), "A comparison of diethyldithiocarbamate and EDTA as antidotes for acute cadmium intoxication." Res Commun Chem Pathol Pharmacol 38(2): 271-8; Pages, A., J.S. Casas, et al. (1985), "Dithiocarbamates in heavy metal poisoning: complexes of N,N-di(1-hydroxyethyl)dithiocarbamate with Zn(II), Cd(II), Hg(II), CH₃Hg(II), and C₆H₅Hg(II).: J. Inorg Biochem 25(1): 35-42; Tandon, S.K., N.S. Hashmi, et al. (1990), "The lead-chelating effects of substituted dithiocarbamates." Biomed Environ Sci 3(3): 299-305.
- Dithiocarbamates have also been used adjunctively in cis-platinum chemotherapy to prevent renal toxicity. Hacker, M.P., W.B. Ershler, et al. (1982). "Effect of disulfiram (tetraethylthiuram disulfide) and diethyldithiocarbamate on the bladder toxicity and antitumor activity of cyclophosphamide in mice." Cancer Res 42(11): 4490-4. Bodenner, 1986 #733; Saran, M. and Bors, W. (1990). "Radical reactions in vivo--an overview." Radiat. Environ. Biophys. 29(4):249-62.

-35-

A dithiocarbamate currently used in the treatment of alcohol abuse is disulfiram, a dimer of diethyldithiocarbamate. Disulfiram inhibits hepatic aldehyde dehydrogenase. Inoue, K., and Fukunaga, et al., (1982). "Effect of disulfiram and its reduced metabolite, diethyldithiocarbamate on aldehyde dehydrogenase of human erythrocytes." Life Sci 30(5): 419-24.

It has been reported that dithiocarbamates inhibit HIV virus replication, and also enhance the maturation of specific T cell subpopulations. This has led to clinical trials of diethyldithiocarbamate in AIDs patient populations. Reisinger, E., et al., (1990). "Inhibition of HIV progression by dithiocarb." Lancet 335: 679.

Dithiocarboxylates are compounds of the structure A-SC(S)-B, which are members of the general class of compounds known as thiol antioxidants, and are alternatively referred to as carbodithiols or carbodithiolates. It appears that the -SC(S)- moiety is essential for therapeutic activity, and that A and B can be any group that does not adversely affect the efficacy or toxicity of the compound.

In an alternative embodiment, one or both of the sulfur atoms in the dithiocarbamate is replaced with a selenium atom. The substitution of sulfur for selenium may decrease the toxicity of the molecule in certain cases, and may thus be better tolerated by the patient.

A and B can be selected by one of ordinary skill in the art to impart desired characteristics to the compound, including size, charge, toxicity, and degree of stability, (including stability in an acidic environment such as the stomach, or basic environment such as the intestinal tract). The selection of A and B will also have an important

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effect on the tissue-distribution and pharmacokinetics of the compound. In general, for treatment of cardiovascular disease, it is desirable that the compound accumulate, or
5 localize, in the arterial intimal layer containing the vascular endothelial cells. The compounds are preferably eliminated by renal excretion.

An advantage in administering a dithio-carboxylate pharmaceutically is that it does not
10 appear to be cleaved enzymatically in vivo by thioesterases, and thus may exhibit a prolonged halflife in vivo.

In a preferred embodiment, A is hydrogen or a pharmaceutically acceptable cation, including but
15 not limited to sodium, potassium, calcium, magnesium, aluminum, zinc, bismuth, barium, copper, cobalt, nickel, or cadmium; a salt-forming organic acid, typically a carboxylic acid, including but not limited to acetic acid, oxalic acid, tartaric
20 acid, succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, naphthalenedisulfonic acid, or polygalacturonic acid; or a cation formed from ammonia or other
25 nitrogenous base, including but not limited to a nitrogenous heterocycle, or a moiety of the formula $NR^4R^5R^6R^7$, wherein R^4 , R^5 , R^6 , and R^7 are independently hydrogen, C_{1-6} linear, branched, or (in the case of C_{4-6}) cyclic alkyl, hydroxy(C_{1-6})alkyl (wherein one or
30 more hydroxyl groups are located on any of the carbon atoms), or aryl, N,N-dibenzyl-ethylenediamine, D-glucosamine, choline, tetraethylammonium, or ethylenediamine.

In another embodiment, A can be a
35 physiologically cleavable leaving group that can be cleaved in vivo from the molecule to which it is attached, and includes but is not limited acyl

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(including acetyl, propionyl, and butyryl), alkyl, phosphate, sulfate or sulfonate.

In one embodiment, B is alkyl, alkenyl, alkynyl, alkaryl, aralkyl, haloalkyl, haloalkenyl, haloalkynyl, aryl, alkaryl, hydrogen, C₁₋₆ alkoxy-C₁₋₁₀ alkyl, C₁₋₆ alkylthio-C₁₋₁₀ alkyl, NR²R³, -(CHOH)_nCH₂OH, wherein n is 0, 1, 2, 3, 4, 5, or 6, -(CH₂)_nCO₂R¹, including alkylacetyl, alkylpropionyl, and alkylbutyryl, or hydroxy(C₁₋₆)alkyl- (wherein one or more hydroxyl groups are located on any of the carbon atoms).

In another embodiment, B is NR²R³, wherein R² and R³ are independently alkyl; -(CHOH)_n(CH₂)_nOH, wherein n is 0, 1, 2, 3, 4, 5, or 6; -(CH₂)_nCO₂R¹, -(CH₂)_nCO₂R⁴; hydroxy(C₁₋₆)alkyl-; alkenyl (including but not limited to vinyl, allyl, and CH₃CH=CH-CH₂CH₂); alkyl(CO₂H), alkenyl(CO₂H), alkynyl(CO₂H), or aryl, wherein the aryl group can be substituted as described above, notably, for example, with a NO₂, CH₃, t-butyl, CO₂H, halo, or p-OH group; or R² and R³ can together constitute a bridge such as -(CH₂)_m-, wherein m is 3, 4, 5, or 6, and wherein R⁴ is alkyl, aryl, alkaryl, or aralkyl, including acetyl, propionyl, and butyryl.

In yet another embodiment, B can be a heterocyclic or alkylheterocyclic group. The heterocycle can be optionally partially or totally hydrogenated. Nonlimiting examples are those listed above, including phenazine, phenothiazine, pyridine and dihydropyridine.

In still another embodiment, B is the residue of a pharmaceutically-active compound or drug. The term drug, as used herein, refers to any substance used internally or externally as a medicine for the treatment, cure, or prevention of a disease or disorder.

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Nonlimiting examples are drugs for the treatment or prevention of cardiovascular disease, including antioxidants such as probucol; nicotinic acid; agents that prevent platelets from sticking, such as aspirin; antithrombotic agents such as coumadin; calcium channel blockers such as verapamil, diltiazem, and nifedipine; angiotensin converting enzyme (ACE) inhibitors such as captopril and enalapril, β -blockers such as propranolol, terbutalol, and labetalol, nonsteroidal antiinflammatories such as ibuprofen, indomethacin, fenoprofen, mefenamic acid, flufenamic acid, sulindac, or corticosteroids. The -C(S)SA group can be directly attached to the drug, or attached through any suitable linking moiety.

In another embodiment, the dithiocarbamate is an amino acid derivative of the structure $AO_2C-R^9-NR^{10}-C(S)SA$, wherein R_9 is a divalent B moiety, a linking moiety, or the internal residue of any of the naturally occurring amino acids (for example, CH_3CH for alanine, CH_2 for glycine, $CH(CH_2)_4NH_2$ for lysine, etc.), and R^{10} is hydrogen or lower alkyl.

B can also be a polymer to which one or more dithiocarbamate groups are attached, either directly, or through any suitable linking moiety. The dithiocarbamate is preferably released from the polymer under in vivo conditions over a suitable time period to provide a therapeutic benefit. In a preferred embodiment, the polymer itself is also degradable in vivo. The term biodegradable or bioerodible, as used herein, refers to a polymer that dissolves or degrades within a period that is acceptable in the desired application (usually in vivo therapy), usually less than five years, and preferably less than one year, on exposure to a physiological solution of pH 6-8 having a temperature of between 25 and 37°C. In a preferred

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embodiment, the polymer degrades in a period of between 1 hour and several weeks, according to the application.

A number of degradable polymers are known.

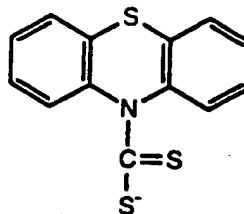
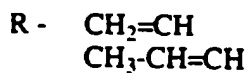
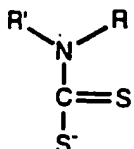
- 5 Nonlimiting examples are peptides, proteins, nucleoproteins, lipoproteins, glycoproteins, synthetic and natural polypeptides and polyamino acids, including but not limited to polymers and copolymers of lysine, arginine, asparagine, 10 aspartic acid, cysteine, cystine, glutamic acid, glutamine, hydroxylysine, serine, threonine, and tyrosine; polyorthoesters, including poly(α -hydroxy acids), for example, polylactic acid, polyglycolic acid, poly(lactide-co-glycolide); polyanhydrides, 15 albumin or collagen, a polysaccharide containing sugar units such as lactose, and polycaprolactone. The polymer can be a random or block copolymer.

- B can also be a group that enhances the water solubility of the dithiocarbamate, for example, 20 -lower alkyl-O-R⁸, wherein R⁸ is -PO₂(OH)M⁺ or PO₃(M⁺)₂, wherein M⁺ is a pharmaceutically acceptable cation; -C(O)(CH₂)₂CO₂⁻M⁺, or -SO₃M⁺; -lower alkylcarbonyl-lower alkyl; -carboxy lower alkyl; -lower alkylamino-lower alkyl; N,N-di-substituted amino 25 lower alkyl-, wherein the substituents each independently represent lower alkyl; pyridyl-lower alkyl-; imidazolyl-lower alkyl-; imidazolyl-Y-lower alkyl wherein Y is thio or amino; morpholinyl-lower alkyl; pyrrolidinyl-lower alkyl; thiazolinyl-lower alkyl-; piperidinyl-lower alkyl; morpholinyl-lower 30 hydroxyalkyl; N-pyrrol; piperazinyl-lower alkyl; N-substituted piperazinyl-lower alkyl, wherein the substituent is lower alkyl; triazolyl-lower alkyl; tetrazolyl-lower alkyl; tetrazolylamino-lower 35 alkyl; or thiazolyl-lower alkyl.

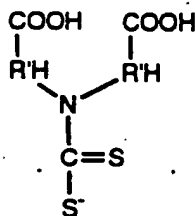
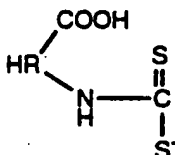
In an alternative embodiment, a dimer such as B-C(S)S-SC(S)-B can be administered.

Nonlimiting examples of dithiocarbamates are those of the structure:

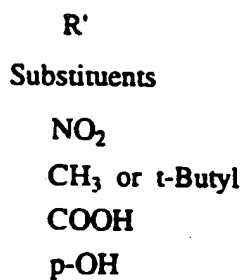
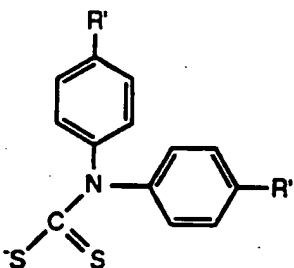
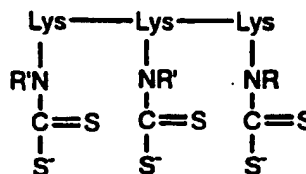
1) Aliphatic Substrate



2) Amino Acid



Polyamino acid



R -

R'

Na⁺

Ca⁺⁺

NH₄⁺

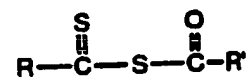
Choline⁺ and quaternary amines

Mg⁺⁺

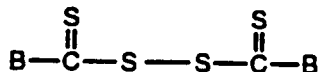
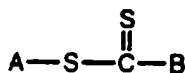
Al⁺⁺⁺

K⁺

H⁺



Thioester



Dithiocarboxylates should be chosen for use in treating atherosclerosis and other cardiovascular and inflammatory diseases that have the proper lipophilicity to locate at the affected site. The compound should not compartmentalize in low turnover regions such as fat deposits. In a preferred embodiment for treatment of cardiovascular disease, the pharmacokinetics of the compound should not be dramatically affected by congestive heart failure or renal insufficiency.

For topical applications for the treatment of inflammatory skin disorders, the selected compound should be formulated to be absorbed by the skin in a sufficient amount to render a therapeutic effect to the afflicted site.

The dithiocarboxylate must be physiologically acceptable. In general, compounds with a therapeutic index of at least 2, and preferably at least 5 or 10, are acceptable. The therapeutic index is defined as the EC_{50}/IC_{50} , wherein EC_{50} is the concentration of compound that inhibits the expression of VCAM-1 by 50% and IC_{50} is the concentration of compound that is toxic to 50% of the target cells. Cellular toxicity can be measured by direct cell counts, trypan blue exclusion, or various metabolic activity studies such as 3H-thymidine incorporation, as known to those skilled in the art. The therapeutic index of PDTC in tissue culture is over 100 as measured by cell toxicity divided by ability to inhibit VCAM-1 expression activated by TNF α , in HUVE cells. Initial studies on the rapidly dividing cell type HT-18 human glioma demonstrate no toxicity at concentrations 100-fold greater than the therapeutic concentration. Disulfiram, an orally administered form of diethyldithiocarbamate, used in the treatment of alcohol abuse, generally

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elicits no major clinical toxicities when administered appropriately.

There are a few dithiocarbamates that are known to be genotoxic. These compounds do not fall within the scope of the present invention, which is limited to the administration of physiologically acceptable materials. An example of a genotoxic dithiocarbamate is the fungicide zinc dimethyldithiocarbamate. Further, the anticholinesterase properties of certain dithiocarbamates can lead to neurotoxic effects. Miller, D. (1982). Neurotoxicity of the pesticidal carbamates. Neurobehav. Toxicol. Teratol. 4(6): 779-87.

The term dithiocarboxylate as used herein specifically includes, but is not limited to, dithiocarbamates of the formulas:



wherein R^1 is H or a pharmaceutically acceptable cation, including but not limited to sodium, potassium, or $NR^4R^5R^6R^7$, wherein R^4 , R^5 , R^6 , and R^7 are independently hydrogen, C_{1-6} linear, branched, or cyclic alkyl, hydroxy(C_{1-6})alkyl (wherein one or more hydroxyl groups are located on any of the carbon atoms), or aryl, and

R^2 and R^3 are independently C_{1-10} linear, branched or cyclic alkyl; $-(CHOH)_n(CH_2)_mOH$, wherein n is 0, 1, 2, 3, 4, 5, or 6; $-(CH_2)_nCO_2R^1$, $-(CH_2)_nCO_2R^4$; hydroxy(C_{1-6})alkyl-, or R^2 and R^3 together constitute a bridge such as $-(CH_2)_m-$, wherein m is 3-6, and wherein R^4 is alkyl, aryl, alkaryl, or aralkyl, including acetyl, propionyl, and butyryl.

Specific examples of useful dithiocarbamates, illustrated in Figure 15, include sodium pyrrolidine-N-carbodithioate, sodium N-methyl-N-carboxymethyl-N-carbodithioate, trisodium N,N-di(carboxymethyl)-N-carbodithioate, sodium N-

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methyl-D-glucamine-N-carbodithioate, sodium N,N-diethyl-N-carbodithioate (sodium diethyldithiocarbamate), and sodium N,N-diisopropyl-N-carbodithioate.

- 5 The active dithiocarboxylates and in particular, dithiocarbamates are either commercially available or can be prepared using known methods.

II. Biological Activity

- 10 The ability of dithiocarboxylates to inhibit the expression of VCAM-1 can be measured in a variety of ways, including by the methods set out in detail below in Examples 9 to 15. For convenience, Examples 9-11 and 14-15 describe the evaluation of the biological activity of sodium pyrrolidine-N-carbodithioate (also referred to as PDTC). These
15 examples are not intended to limit the scope of the invention, which specifically includes the use of any of the above-described compounds to treat atherosclerosis, and other types of inflammation
20 and cardiovascular disease mediated by VCAM-1. Any of the compounds described above can be easily substituted for PDTC and evaluated in similar fashion.

- Examples 12 and 13 provide comparative data on
25 the ability of a number of dithiocarbamates to inhibit the gene expression of VCAM-1. The examples below establish that the claimed dithiocarbamates specifically block the ability of VCAM-1 to be expressed by vascular endothelial
30 cells in response to many signals known to be active in atherosclerosis and the inflammatory response.

Experimental Procedures

- Cell Cultures HUVE cells were isolated from
35 human umbilical veins that were cannulated,

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perfused with Hanks solution to remove blood, and then incubated with 1% collagenase for 15 minutes at 37°C. After removal of collagenase, cells were cultured in M199 medium supplemented with 20% fetal bovine serum (HyClone), 16 µg/ml heparin (ESI Pharmaceuticals, Cherry Hill, NJ), 50 µg/ml endothelial cell growth supplement (Collaborative Research Incorporated, Bedford MA), 25 mM Hepes Buffer, 2 mM L-glutamin, 100 µg/ml penicillin and 100 µg/ml streptomycin and grown at 37°C on tissue culture plates coated 0.1% gelatin. Cells were passaged at confluency by splitting 1:4. Cells were used within the first 8 passages.

Incubation with Cytokines and Other Reagents

Confluent HUVE cells were washed with phosphate buffered saline and then received fresh media. The indicated concentrations of PDTC were added as pretreatment 30 minutes before adding cytokines. Cytokines and other inducers were directly added to medium for the times and at the concentrations indicated in each experiment. Human recombinant IL-1b was the generous gift of Upjohn Company (Kalamazoo, Michigan). TNFa was obtained from Bohringer Engelheim. Bacterial lipopolysaccharide (LPS), polyinosinic acid: polycitidilic acid (Poly I:C), and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma Chemical (St. Louis, MO). All other reagents were of reagent grade.

RNA Isolation: Total cellular RNA was isolated by a single extraction using an acid guanidium thiocyanate-phenol-chloroform mixture. Cells were rinsed with phosphate buffered saline and then lysed with 2 ml of guanidium isothiocyanate. The solution was acidified with 0.2 ml of sodium acetate (pH 4.0) and then extracted with 2 ml phenol and 0.4 ml chloroform:isoamyl alcohol (24:1). The RNA underwent two ethanol

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precipitations prior to being used for Northern blot analysis.

Northern Blot Analysis: Total cellular RNA (20 µg) was size fractionated using 1% agarose formaldehyde gels in the presence of 1 µg/ml ethidium bromide. The RNA was transferred to a nitrocellulose filter and covalently linked by ultraviolet irradiation using a Stratlinker UV crosslinker (Stratagene, La Jolla, CA).

Hybridizations were performed at 42°C for 18 hours in 5X SSC (1X=150 mM NaCl, 15 mM Na citrate), 1% sodium dodecyl sulfate, 5X Denhardt solution, 50% formamide, 10% dextran sulfate and 100 µg/ml of sheared denatured salmon sperm DNA. Approximately 1-2X 10⁶ cpm/ml of labeled probe (specific activity > 10⁸ cpm/µg DNA) were used per hybridization. Following hybridization, filters were washed with a final stringency of 0.2X SSC at 55°C. The nitrocellulose was stripped using boiled water prior to rehybridization with other probes. Autoradiography was performed with an intensifying screen at -70°C.

³²P probes: ³²P labeled DNA probes were made using the random primer oligonucleotide method. The ICAM-1 probe was an Eco R1 fragment of human cDNA. The ELAM-1 probe was a 1.85 kb Hind III fragment of human cDNA. The VCAM-1 probe was a Hind III-Xho I fragment of the human cDNA consisting of nucleotide 132 to 1814.

Enzyme Linked Immunosorbent Assay (ELISA): HUVE cells were plated on 96-well tissue culture plates 48 to 72 hours before the assay. Primary antibodies in M199 with 5% FBS were added to each well and incubated one hour at 37°C. The cells were then washed and incubated for one hour with peroxidase conjugated goat anti-mouse IgG (Bio Rad) diluted 1/500 in M199 with 5% FBS. The wells were

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then washed and binding of antibody was detected by the addition of 100 μ l of 10 mg/ml 3,3',5,5'-tetramethyl-benzidine (Sigma) with 0.003% H_2O_2 . The reaction was stopped by the addition of 25 μ l of 8N sulfuric acid. Plates were read on an ELISA reader (Bio Rad) at OD 450 nm after blanking on rows stained only with second step antibody. Data represent the means of triplicate.

Antibodies: Monoclonal antibody (MAb) 4B9 recognizing vascular cell adhesion molecule-1 (VCAM-1) was the generous gift of Dr. John Harlan (University of Washington). MAb E9A1F1 recognizing endothelial cell adhesion molecule (ELAM-1) was the generous gift of Dr. Swerlick (Emory University). Hybridomas producing mAb 84H10 recognizing intercellular adhesion molecule 1 (ICAM-1) are routinely grown in our laboratory and antibody was used as tissue culture supernatant.

Example 9 PDTC Blocks IL-1b Mediated Induction of HUVEC VCAM-1, but not ICAM-1 or ELAM-1, mRNA Accumulation

To determine whether the oxidative state of the endothelial cell can alter the basal or induced expression of cell adhesion molecule genes, cultured human vascular endothelial cells were exposed to the inducing cytokine, IL-1b (10 U/ml) in the presence or absence of the thiolated metal chelating antioxidant, pyrrolidine dithiocarbamate (PDTC, 50 μ M) for up to 24 hours. As shown in Figure 10, IL-1b alone (lanes 2, 4, 6, 8) induces the expected rapid and transient induction of VCAM-1 (Panel A), E-selectin (ELAM-1, Panel B) and ICAM-1 (Panel C) mRNA accumulation, all of which peak at four hours. However, in the presence of PDTC, IL-1b induction of VCAM-1 mRNA accumulation is dramatically inhibited by over 90% (panel A, lanes 3, 5, 7, 9). In contrast, although IL-1b mediated

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induction of ELAM-1 is slightly inhibited at 2 and 24 hours (compare lane 2 and 3, 8 and 9, panel B), PDTC does not inhibit the induction at 4 and 8 hours (lane 5 and 7, panel B). IL-1b mediated induction of ICAM-1 mRNA accumulation is not affected (panel B, lanes 3, 5, 7, 9). Indeed, a mild augmentation of IL-1b induction of ICAM-1 mRNA accumulation (~30%) is observed (compare lanes 4 and 5, panel B). Equivalent amounts of nitrocellulose transferred RNA per lane was confirmed by ethidium bromide staining and visualization.

A dose-response analysis was performed to determine whether PDTC inhibits the induction of VCAM-1 gene expression by IL-1b in a dose dependent manner. As shown in Figure 11, PDTC inhibits IL-1b mediated induction of VCAM-1 gene expression with a steep dose-response curve (Figure 11, panel A) with a calculated EC_{50} of approximately 10 μ M, while PDTC does not inhibit IL-1b mediated induction of ELAM-1 expression with these concentrations (Fig. 11, panel B). The IL-1b mediated induction of ICAM-1 mRNA accumulation is enhanced by PDTC with the concentration higher than 0.5 μ M (Fig. 2, compare lane 2 and lane 4-7, panel C).

These data demonstrate that IL-1b utilizes a dithiocarboxylate, and in particular, a dithiocarbamate sensitive step as part of its signaling mechanism in the induction of VCAM-1 gene expression. The data also appear to indicate that this dithiocarbamate sensitive step does not play a significant role in the IL-1b mediated induction of ELAM-1 or ICAM-1 gene expression.

Example 10 PDTC Blocks Induction of HUVEC VCAM-1 mRNA Accumulation by Multiple Stimuli

To determine whether other well-described activators of VCAM-1 gene expression also utilize a

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PDTC sensitive step, three distinct classes of activators were tested: another classic receptor mediated inducing agent (TNFa), a non-receptor mediated inducer (lipopolysaccharide (LPS)) and a recently described novel inducer (double stranded RNA, poly(I:C)). In all three cases, PDTC dramatically inhibited the induction of VCAM-1 mRNA accumulation in HUVECs after four hours (Figure 12, Panel A). Although the TNFa mediated ELAM-1 gene expression is suppressed to some extent (Fig. 12 lane 1 and 2, panel B), LPS and poly(I:C) mediated ELAM-1 mRNA accumulation was unaffected (Fig. 12 lane 3-6, panel B). The induction of ICAM-1 mRNA accumulation was unaffected (Figure 12, Panel C). This data indicates that structurally distinct inducing agents, acting through distinct pathways, share a common regulatory step specific for the induction of VCAM-1 gene expression.

Example 11 PDTC Blocks HUVE Cell Surface Expression of VCAM-1 Induced by Multiple Stimuli

To determine whether, like its mRNA, the induction of endothelial cell surface protein expression of VCAM-1 could also be inhibited by PDTC, monoclonal antibodies were used in an ELISA assay to quantitate the induction of cell surface VCAM-1 and ICAM-1 in cultured HUVE cells. As shown in Figure 13, multiple classes of activating agents, in the absence of PDTC (-PDTC), induce the rapid and transient accumulation of VCAM-1 (top left panel) at the cell surface peaking at six hours. In the presence of PDTC (+PDTC, top right panel), the induction of cell surface expression of VCAM-1 by all agents tested is dramatically inhibited (80-90%). In contrast, the induced expression of cell surface ICAM-1 is unaffected

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under identical conditions (bottom left and right panels).

These data demonstrate that, like its mRNA accumulation, cell surface VCAM-1 expression are selectively inhibited by dithiocarbamates and that multiple classes of activating agents utilize a similar, dithiocarbamate sensitive mechanism to induce VCAM-1 gene expression.

10 **Example 12 Comparative Effectiveness of Antioxidants in Blocking TNF α Induction of VCAM-1**

To determine whether structurally similar or dissimilar antioxidants could also inhibit VCAM-1 gene expression, and with what potency, HUVE cells were exposed to TNF α for six hours in the presence or absence of different concentrations of four different antioxidants. As shown in Figure 14, both diethyldithiocarbamate (DETC) and N-acetyl cysteine (NAC) inhibited VCAM-1 expression at concentrations of 5 μ M and 30 μ M, respectively. In contrast, PDTC (PDTC) was effective between 5 and 50 μ M. The iron metal chelator, desferroximine, had no effect at the concentrations tested.

25 **Example 13 PDTC Inhibits TNF Induction of VCAM-1/VLA-4 Mediated Adhesion**

The ability of a variety of antioxidants to inhibit TNF- α induction of VCAM-1 in HUVE cells was evaluated by the method set out in Example 12. Figure 15 is a graph of the relative VCAM-1 cell surface expression (O.D. 595 nm) in TNF- α activated HUVE cells versus concentrations of PTDC (sodium N-pyrrolidine dithiocarbamate), DIDTC (sodium N,N-diethyl-N-carbodithioate), SarDTC (sodium N-methyl-N-carboxymethyl-N-carbodithioate), IDADTC (trisodium N,N-di(carboxymethyl)-N-carbodithioate),

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MGDTC (sodium N-methyl-D-glucamine-N-carbodithioate), MeOBGDTC (sodium N-(4-methoxybenzyl)-D-glucamine-N-carbodithioate), DEDTC (sodium N,N-diethyl-N-carbodithioate), Di-PDTC (sodium N,N-diisopropyl-N-carbodithioate), and NAC is (N-acetyl cysteine).

Example 13 PDTC Inhibits TNF Induction of VCAM-1/VLA-4 Mediated Adhesion

In order to define whether PDTC inhibition of VCAM-1 regulation is associated with functional consequences, the binding of Molt-4 cells to HUVEC cells either unstimulated or stimulated with TNFa (100U/ml) was examined for six hours in the presence or absence of PDTC. Molt-4 cells have been previously shown to bind to activated HUVEC via a VCAM-1 dependent mechanism. As shown in Figure 16, the percentage of Molt-4 binding to HUVEC cells decreased when PDTC was present in the media.

Example 14 PDTC Inhibits Monocyte Binding to the Thoracic Aorta of Cholesterol Fed Rabbits

An experiment was performed to determine whether the thiol antioxidant PDTC would be efficacious in blocking the first monocyte binding component of atherosclerosis in an experimental animal model. One mature New Zealand white rabbit (3.5 Kg) received an intravenous injection of PDTC (20 mg/Kg, as a concentration of 20 mg/ml in PBS) once daily for 5 days. Injections were given via an indwelling cannula in the marginal ear vein, which as kept patent by flushing with heparinized saline solution. The PDTC solution was mixed fresh daily or on alternate days (stored light-protected at 4°C), and filtered (0.45 mm pore filter) just prior to use. After the first injection, when the

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cannula was placed, the drug was administered with the rabbit in the conscious state without apparent discomfort or other ill effect. On the second day of injections, the rabbit was given chow containing 1% cholesterol by weight, which was continued throughout the remainder of the experiment. On the fifth day, the animal was euthanized and the thoracic aorta was excised and fixed. After appropriate preparation, the sample was imaged on the lower stage of an ISI DS-130 scanning electron microscope equipped with a LaB emitter. Using dual-screen imaging and a transparent grid on the CRT screen, 64 adjacent fields at a 620x magnification were assessed, to cover an area of ~1.3 mm². Within each field, the number of adherent leukocytes (WBC) and erythrocytes (RBC) were counted and recorded.

The data from the arch sample are as follows: 5 WBC and ~25 RBC per 1.3 mm² field. This level of WBC adhesion is similar to control animals fed regular chow (about 7 per field have been seen in arch and thoracic samples from 2 'negative control' experiments). 'Positive control' rabbits fed 1% cholesterol for 4 days but not given antioxidant show about a 5-fold increase in adhesion, to 38 WBC/1.3 mm². A considerable amount of mostly cell-sized debris was observed adherent to each arch sample. It is unclear whether this material is an artifact of preparation, or was present in vivo, and if so, whether it is related to PDTC administration. These studies suggest that PDTC infusions can effectively block initial monocyte adhesion to the aortic endothelium.

Example 15 Inhibition of BSA 13-HPODE Adducts with PDTC

Figure 18 is a bar chart graph of the effect of PTDC on the formation of fluorescent adducts of BSA

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and 13-HPODE, as measured in fluorescent units versus micromolar concentration of PDTC. One micromolar of 13-HPODE was incubated with 200 micrograms of BSA in the presence of PDTC for six
5 days. Fluorescence was measured at 430-460 nm with excitation at 330-360 nm. For details of the assay, see Freebis, J., Parthasarathy, S., Steinberg, D, Proceedings of the National Academy of Sciences 89, 10588-10592, 1992. In a typical
10 reaction 100 nmols of LOOH (generated by the lipoxxygenase catalyzed oxidation of linoleic acid) in incubated with 100 μ g of bovine serum albumin for 48 to 72 hours and the formation of fluorescent products are followed by measuring the fluorescent
15 spectrum with excitation at 360 nm and emission between 390 and 500 nm.

As indicated, PDTC decreases the concentration of fluorescent adducts of BSA and 13-HPODE.

Figure 19 is a graph of the effect of PTDC on
20 the formation of fluorescent adducts of BSA and ox-PUFA as a function of wavelength (nm) and concentration of PDTC. As the concentration of PDTC increases, the quantity of fluorescent adducts decrease.

25 **Example 16 Effect of PDTC on the oxidation of LDL by horseradish peroxidase**

Figure 20 is a graph of the effect of PDTC on the oxidation of LDL by horseradish peroxidase (HRP), as measured over time (minutes) for varying
30 concentrations of PDTC. The oxidation of LDL was followed by measuring the oxidation of the fatty acid components of LDL as determined by the increase in optical density at 234 nm. When a polyunsaturated fatty acid is oxidized, there is a
35 shift of double bonds resulting in the formation of conjugated dienes which absorb at 234 nm. The

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intercept of the initiation and propagation curve (lag phase) is suggested to be a measure of the oxidizability of LDL. Higher the lag phase, more resistant is the LDL to oxidation. Typically 100
5 μ g of human LDL is incubated with 5 μ M H_2O_2 and the increase in absorption of 234 nm is followed.

It is observed that after an incubation period, PDTC inhibits the oxidation of LDL by HRP in a manner that is concentration dependent.

10 **Example 17 Effect of PDTC on the cytokine-induced formation of ox-PUFA**

Figure 21 is a chart of the effect of PDTC on the cytokine-induced formation of ox-PUFA in human aortic endothelial cells. As indicated, both TNF- α
15 and IL-1 β causes the oxidation of linoleic acid to ox-linoleic acid. The oxidation is significantly prevented by PDTC.

2. Modification of the Synthesis and Metabolism of PUFAs and ox-PUFAs

20 Inhibition of the expression of VCAM-1 can be accomplished via a modification of the metabolism of PUFAs into ox-PUFAs. For example, a number of enzymes are known to oxidize unsaturated materials, including peroxidases, lipoxygenases,
25 cyclooxygenases, and cytochrome P450. The inhibition of these enzymes may prevent the oxidation of PUFAs in vivo. PUFAs can also be oxidized by metal-dependent nonenzymatic materials.

30 **IV. Method for Modifying the Expression of a Redox-Sensitive Gene**

In an alternative embodiment, a method is provided for suppressing the expression of a redox-sensitive gene or activating a gene that is

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suppressed through a redox-sensitive pathway, that includes administering an effective amount of a substance that prevents the oxidation of the oxidized signal, and typically, the oxidation of a polyunsaturated fatty acid. Representative redox-sensitive genes that are involved in the presentation of an immune response include, but are not limited to, those expressing cytokines involved in initiating the immune response (e.g., IL-1 β), chemoattractants that promote the migration of inflammatory cells to a point of injury (e.g., MCP-1), growth factors (IL-6, thrombin receptor), and adhesion molecules (e.g., VCAM-1 and E-selectin).

Given this disclosure, one of ordinary skill in the art will be able to screen a wide variety of antioxidants for their ability to suppress the expression of a redox-sensitive gene or activate a gene that is suppressed through a redox-sensitive pathway. All of these embodiments are intended to fall within the scope of the present invention.

Based on the results of this screening, nucleic acid molecules containing the 5' regulatory sequences of the redox-sensitive genes can be used to regulate or inhibit gene expression *in vivo* can be identified. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5' flanking region-gene construct in cells depending on the preference and judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors are being developed that enable the introduction of nucleic acid sequences *in vivo* (see, e.g., Mulligan, 1993 Science, 260, 926-932; United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference). Recently, a

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delivery system was developed in which nucleic acid is encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, e.g., Zhu et al., 1993 Science 261, 209-211; incorporated herein by reference).

The 5' flanking sequences of the redox-sensitive gene can be used to inhibit the expression of the redox-sensitive gene. For example, an antisense RNA of all or a portion of the 5' flanking region of the redox-sensitive gene can be used to inhibit expression of the gene *in vivo*. Expression vectors (e.g., retroviral expression vectors) are already available in the art which can be used to generate an antisense RNA of a selected DNA sequence which is expressed in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286).

Accordingly, DNA containing all or a portion of the sequence of the 5' flanking region of the gene can be inserted into an appropriate expression vector so that upon passage into the cell, the transcription of the inserted DNA yields an antisense RNA that is complementary to the mRNA transcript of the gene normally found in the cell. This antisense RNA transcript of the inserted DNA can then base-pair with the normal mRNA transcript found in the cell and thereby prevent the mRNA from being translated. It is of course necessary to select sequences of the 5' flanking region that are downstream from the transcriptional start sites for the redox-sensitive gene to ensure that the antisense RNA contains complementary sequences present on the mRNA. Antisense RNA can be generated *in vitro* also, and then inserted into cells. Oligonucleotides can be synthesized on an

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automated synthesizer (e.g., Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have been shown to be

5 effective in inhibiting gene transcription and viral replication (see e.g., Zamecnik et al., 1978 Proc. Natl. Acad. Sci. USA 75, 280-284; Zamecnik et al., 1986 Proc. Natl. Acad. Sci., 83, 4143-4146; Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA

10 85, 1028-1032; Crooke, 1993 FASEB J. 7, 533-539. Furthermore, recent work has shown that improved inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see,

15 e.g., Offensperger et al., 1993 EMBO J. 12, 1257-1262 (in vivo inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides); Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al., 1988 Proc. Natl.

20 Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., 1989 Proc.

25 Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of antisense methylphosphonate oligonucleotides); Shaw et al., 1991 Nucleic Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal

30 phosphoroamidate modifications); incorporated herein by reference).

The sequences of the 5' flanking region of the redox-sensitive gene can also be used in triple helix (triplex) gene therapy. Oligonucleotides

35 complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local

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triple nucleic acid helices which block transcription of the gene (see, e.g., 1989 Maher et al., Science 245, 725-730; Orson et al., 1991 Nucl. Acids Res. 19, 3435-3441; Postal et al., 1991 Proc. Natl. Acad. Sci. USA 88, 8227-8231; Cooney et al., 1988 Science 241, 456-459; Young et al., 1991 Proc. Natl. Acad. Sci. USA 88, 10023-10026; Duval-Valentin et al., 1992 Proc. Natl. Acad. Sci. USA 89, 504-508; 1992 Blume et al., Nucl. Acids Res. 20, 1777-1784; 1992 Grigoriev et al., J. Biol. Chem. 267, 3389-3395.

Recently, both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides for use in oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, e.g., Maher et al., (1989); Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et al., (1991); Holt et al., 1988 Mol. Cell. Biol. 8, 963-973; Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85, 1028-1032). To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992)).

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Methods to produce or synthesize oligonucleotides are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see also, Ikuta et al., in Ann. Rev. Biochem. 1984 53, 323-356 (phosphotriester and phosphite-triester methods); Narang et al., in Methods Enzymol., 65, 610-620 (1980) (phosphotriester method). Accordingly, DNA sequences of the 5' flanking region of the redox-sensitive gene described herein can be used to design and construct oligonucleotides including a DNA sequence consisting essentially of at least 15 consecutive nucleotides, with or without base modifications or intercalating agent derivatives, for use in forming triple helices specifically within the 5' flanking region of a redox-sensitive gene in order to inhibit expression of the gene.

In some cases it may be advantageous to insert enhancers or multiple copies of the regulatory sequences into an expression system to facilitate screening of methods and reagents for manipulation of expression.

V. Models and Screens

Screens for disorders mediated by VCAM-1 or a redox-sensitive gene are also provided that include the quantification of surrogate markers of the disease. In one embodiment, the level of oxidized polyunsaturated fatty acid, or other appropriate markers, in the tissue or blood, for example, of a host is evaluated as a means of assessing the "oxidative environment" of the host and the host's

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susceptibility to VCAM-1 or redox-sensitive gene mediated disease.

In another embodiment, the level of circulating or cell-surface VCAM-1 or other appropriate marker and the effect on that level of administration of an appropriate antioxidant is quantified.

In yet another assay, the sensitization of a host's vascular endothelial cells to polyunsaturated fatty acids or their oxidized counterparts is evaluated. This can be accomplished, for example, by challenging a host with a PUFA or ox-PUFA and comparing the resulting concentration of cell-surface or circulating VCAM-1 or other surrogate marker to a population norm.

In another embodiment, in vivo models of atherosclerosis or other heart or inflammatory diseases that are mediated by VCAM-1 can be provided by administering to a host animal an excessive amount of PUFA or oxidized polyunsaturated fatty acid to induce disease. These animals can be used in clinical research to further the understanding of these disorders.

In yet another embodiment of the invention, compounds can be assessed for their ability to treat disorders mediated by VCAM-1 on the basis of their ability to inhibit the oxidation of a polyunsaturated fatty acid, or the interaction of a PUFA or ox-PUFA with a protein target.

This can be accomplished by challenging a host, for example, a human or an animal such as a mouse, to a high level of PUFA or ox-PUFA and then determining the therapeutic efficacy of a test compound based on its ability to decrease circulating or cell surface VCAM-1 concentration. Alternatively, an in vitro screen can be used that is based on the ability of the test compound to prevent the oxidation of a PUFA, or the interaction

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of a PUFA or ox-PUFA with a protein target in the presence of an oxidizing substance such as a metal, for example, copper, or an enzyme such as a peroxidase, lipoxygenase, cyclooxygenase, or cytochrome P450.

In another embodiment, vascular endothelial cells are exposed to TNF- α or other VCAM-1 inducing material for an appropriate time and then broken by any appropriate means, for example by sonication or freeze-thaw. The cytosolic and membrane compartments are isolated. Radiolabeled PUFA is added to defined amounts of the compartments. The ability of the liquid to convert PUFA to ox-PUFA in the presence or absence of a test compound is assayed. Intact cells can be used in place of the broken cell system.

III. Pharmaceutical Compositions

Humans, equine, canine, bovine and other animals, and in particular, mammals, suffering from cardiovascular disorders, and other inflammatory conditions mediated by VCAM-1 or a redox sensitive gene can be treated by administering to the patient an effective amount of a compound that causes the removal, decrease in the concentration of, or prevention of the formation of an oxidized polyunsaturated fatty acids, including but not limited to oxidized linoleic ($C_{18} \Delta^{9,12}$), linolenic ($C_{18} \Delta^{6,9,12}$), arachidonic ($C_{20} \Delta^{5,8,11,14}$) and eicosatrienoic ($C_{20} \Delta^{8,11,14}$) acids; other oxidation signal; or other active compound, or a pharmaceutically acceptable derivative or salt thereof in a pharmaceutically acceptable carrier or diluent. The active materials can be administered by any appropriate route, for example, orally,

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parenterally, intravenously, intradermally, subcutaneously, or topically.

As used herein, the term pharmaceutically acceptable salts or complexes refers to salts or
5 complexes that retain the desired biological activity of the above-identified compounds and exhibit minimal undesired toxicological effects. Nonlimiting examples of such salts are (a) acid
10 addition salts formed with inorganic acids (for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like), and salts formed with organic acids such as acetic acid, oxalic acid, tartaric acid,
15 succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, naphthalenedisulfonic acid, and polygalacturonic acid; (b) base addition salts formed with
20 polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, sodium, potassium, and the like, or with an organic cation formed from N,N-dibenzylethylene-diamine, D-glucosamine, ammonium, tetraethylammonium, or ethylenediamine; or (c)
25 combinations of (a) and (b); e.g., a zinc tannate salt or the like.

The active compound or a mixture of the compounds are administered in any appropriate manner, including but not limited to orally and
30 intravenously. General range of dosage for any of the above-mentioned conditions will be from 0.5 to 500 mg/kg body weight with a dose schedule ranging from once every other day to several times a day. Preferred daily dosages are between approximately 1
35 and 3000 mg/patient/day, more preferably between approximately 5 and 500 mg/patient/day, and even

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more preferably, between approximately 25 and 500 mg/patient/day.

5 The active ingredient should be administered to achieve peak plasma concentrations of the active compound of about 0.1 to 100 μ M, preferably about 1-10 μ M. This may be achieved, for example, by the intravenous injection of a solution or formulation of the active ingredient, optionally in saline, or an aqueous medium or administered as a bolus of the active ingredient.

10 The compounds can also be administered directly to the vascular wall using perfusion balloon catheters following or in lieu of coronary or other arterial angioplasty. As an example, 2-5 mL of a physiologically acceptable solution that contains approximately 1 to 500 μ M of the compound or mixture of compounds is administered at 1-5 atmospheres pressure. Thereafter, over the course of the next six months during the period of maximum risk of restenosis, the active compounds are administered through other appropriate routes and dose schedules.

25 Relatively short term treatments with the active compounds are used to cause the "shrinkage" of coronary artery disease lesions that cannot be treated either by angioplasty or surgery. A nonlimiting example of short term treatment is two to six months of a dosage ranging from 0.5 to 500 mg/kg body weight given at periods ranging from once every other day to three times daily.

30 Longer term treatments can be employed to prevent the development of advanced lesions in high-risk patients. A long term treatment can extend for years with dosages ranging from 0.5 to 500 mg/kg body weight administered at intervals ranging from once every other day to three times daily.

The active compounds can also be administered in the period immediately prior to and following coronary angioplasty as a means to reduce or eliminate the abnormal proliferative and inflammatory response that currently leads to clinically significant re-stenosis.

The active compounds can be administered in conjunction with other medications used in the treatment of cardiovascular disease, including lipid lowering agents such as probucol and nicotinic acid; platelet aggregation inhibitors such as aspirin; antithrombotic agents such as coumadin; calcium channel blockers such as varapamil, diltiazem, and nifedipine; angiotensin converting enzyme (ACE) inhibitors such as captopril and enalapril, and β -blockers such as propanalol, terbutalol, and labetalol. The compounds can also be administered in combination with nonsteroidal antiinflammatories such as ibuprofen, indomethacin, fenoprofen, mefenamic acid, flufenamic acid, sulindac. The compound can also be administered with corticosteroids.

The concentration of active compound in the drug composition will depend on absorption, distribution, inactivation, and excretion rates of the drug as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the

claimed composition. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at varying intervals of time.

- 5 Oral compositions will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be
- 10 incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition.
- 15 The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a
- 20 disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such
- 25 as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various
- 30 other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

The active compound or pharmaceutically acceptable salt or derivative thereof can be

35 administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active

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compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

The active compound or pharmaceutically acceptable derivatives or salts thereof can also be administered with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as antibiotics, antifungals, antiinflammatories, or antiviral compounds.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Suitable vehicles or carriers for topical application are known, and include lotions, suspensions, ointments, creams, gels, tinctures, sprays, powders, pastes, slow-release transdermal patches, aerosols for asthma, and suppositories for application to rectal, vaginal, nasal or oral mucosa.

Thickening agents, emollients, and stabilizers can be used to prepare topical compositions. Examples of thickening agents include petrolatum, beeswax, xanthan gum, or polyethylene glycol, humectants such as sorbitol, emollients such as

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mineral oil, lanolin and its derivatives, or squalene. A number of solutions and ointments are commercially available.

5 Natural or artificial flavorings or sweeteners can be added to enhance the taste of topical preparations applied for local effect to mucosal surfaces. Inert dyes or colors can be added, particularly in the case of preparations designed for application to oral mucosal surfaces.

10 The active compounds can be prepared with carriers that protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can
15 be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the
20 art.

If administered intravenously, preferred carriers are physiological saline or phosphate buffered saline (PBS).

25 The active compound can also be administered through a transdermal patch. Methods for preparing transdermal patches are known to those skilled in the art. For example, see Brown, L., and Langer, R., Transdermal Delivery of Drugs, Annual Review of Medicine, 39:221-229 (1988), incorporated herein by
30 reference.

In another embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including
35 implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides,

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polyglycolic acid, collagen, polyorthoesters, and
polylactic acid. Methods for preparation of such
formulations will be apparent to those skilled in
the art. The materials can also be obtained
5 commercially from Alza Corporation and Nova
Pharmaceuticals, Inc.

Liposomal suspensions may also be
pharmaceutically acceptable carriers. These may be
prepared according to methods known to those
10 skilled in the art, for example, as described in
U.S. Patent No. 4,522,811 (which is incorporated
herein by reference in its entirety). For example,
liposome formulations may be prepared by dissolving
appropriate lipid(s) (such as stearyl phosphatidyl
15 ethanolamine, stearyl phosphatidyl choline,
arachadoyl phosphatidyl choline, and cholesterol)
in an inorganic solvent that is then evaporated,
leaving behind a thin film of dried lipid on the
surface of the container. An aqueous solution of
20 the active compound or its monophosphate,
diphosphate, and/or triphosphate derivatives are
then introduced into the container. The container
is then swirled by hand to free lipid material from
the sides of the container and to disperse lipid
25 aggregates, thereby forming the liposomal
suspension.

Modifications and variations of the present
invention will be obvious to those skilled in the
art from the foregoing detailed description of the
30 invention. Such modifications and variations are
intended to come within the scope of the appended

We claim.

1. A method for suppressing the expression of VCAM-1 comprising administering an effective amount of a substance that prevents or minimizes the oxidation of a polyunsaturated fatty acid.

2. A method for suppressing the expression of a redox-sensitive gene comprising administering an effective amount of a substance that prevents or minimizes the oxidation of a polyunsaturated fatty acid.

3. A method for activating a gene that is suppressed by the oxidation of a polyunsaturated fatty acid, comprising administering an effective amount of a substance that prevents or minimizes the oxidation of a polyunsaturated fatty acid.

4. A method for suppressing the expression of VCAM-1 comprising administering an effective amount of a substance that prevents the interaction between a polyunsaturated fatty acid and a protein that mediates the expression of VCAM-1.

5. The method of claims 1-4, wherein the polyunsaturated acid is selected from the group consisting of oxidized linoleic ($C_{18} \Delta^{9,12}$), linolenic ($C_{18} \Delta^{6,9,12}$), arachidonic ($C_{20} \Delta^{5,8,11,14}$) and eicosatrienoic ($C_{20} \Delta^{8,11,14}$) acid.

6. The method of claim 2 or 3, wherein the redox-sensitive gene is selected from the group consisting of those expressing cytokines involved in initiating the immune response (e.g., IL-1 β), chemoattractants that promote the migration of inflammatory cells to a point of injury (e.g., MCP-1), growth factors (e.g., IL-6 and the thrombin receptor), and adhesion molecules (e.g., VCAM-1 and E-selectin).

7. The method of claims 1-4, wherein the substance is pyrrolidine dithiocarbamate, or its pharmaceutically acceptable salt.

8. A method for the prediction or assessment of disorders mediated by VCAM-1 in vivo, comprising quantifying the level of oxidized polyunsaturated fatty acid in the tissue or blood.

9. A method for the prediction or assessment of redox-sensitive gene mediated disease in vivo, comprising quantifying the level of oxidized polyunsaturated fatty acid in the tissue or blood.

11. A method for the prediction or assessment of disorders mediated by VCAM-1 in vivo, comprising quantifying a surrogate marker for the level of oxidized polyunsaturated fatty acid in the tissue or blood.

12. A method for the prediction or assessment of redox-sensitive gene mediated disease in vivo, comprising quantifying a surrogate marker for the level of oxidized polyunsaturated fatty acid in the tissue or blood.

13. The method of claim 11, wherein the surrogate marker is circulating or cell-surface VCAM-1.

14. A method for the evaluation of the sensitization of a host's vascular endothelial cells to polyunsaturated fatty acids or their oxidized counterparts, comprising challenging a host with a PUFA or ox-PUFA and comparing the resulting concentration of cell-surface or circulating VCAM-1 or other surrogate marker to a population norm.

15. A method to screen compounds for their ability to treat disorders mediated by VCAM-1 comprising evaluating the ability of the compound to inhibit the oxidation of a polyunsaturated fatty acid.

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16. A method to screen compounds for their ability to treat disorders mediated by VCAM-1 comprising evaluating the ability of the compound to inhibit the interaction of a PUFA or ox-PUFA with a protein target.

17. A method for the treatment of a cardiovascular disease in humans comprising administering an effective amount of a dithiocarbamate of the formula A-SC(S)-B;

wherein A selected from the group consisting of hydrogen, a pharmaceutically acceptable cation, and a physiologically cleavable leaving group;

and B is selected from the group consisting of alkyl, alkenyl, alkynyl, alkaryl, aralkyl, haloalkyl, haloalkenyl, haloalkynyl, aryl, alkaryl, hydrogen, C₁₋₆ alkoxy-C₁₋₁₀ alkyl, C₁₋₆ alkylthio-C₁₋₁₀ alkyl, NR²R³, -(CHOH)_nCH₂OH, wherein n is 0, 1, 2, 3, 4, 5, or 6, -(CH₂)_nCO₂R¹, including alkylacetyl, alkylpropionyl, and alkylbutyryl, and hydroxy(C₁₋₆)alkyl-.

18. The method of claim 17, wherein A is hydrogen or a pharmaceutically acceptable cation selected from the group consisting of sodium, potassium, calcium, magnesium, aluminum, zinc, bismuth, barium, copper, cobalt, nickel, or cadmium.

19. The method of claim 17, wherein A is a salt-forming organic acid.

20. The method of claim 19, wherein A is selected from the group consisting of choline, acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, naphthalenedisulfonic acid, and polygalacturonic acid.

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21. The method of claim 17, wherein A is a cation formed from ammonia or other nitrogenous base.

22. The method of claim 21, wherein A is a nitrogenous heterocycle, or a moiety of the formula $NR^4R^5R^6R^7$, wherein R^4 , R^5 , R^6 , and R^7 are independently hydrogen, C_{1-6} alkyl, hydroxy(C_{1-6})alkyl, aryl, N,N-dibenzylethylene-diamine, D-glucosamine, tetraethylammonium, or ethylenediamine.

23. The method of claim 17, wherein A is a physiologically cleavable leaving group.

24. The method of claim 17, wherein A is an acyl group.

25. The method of claim 17, wherein B is NR^2R^3 , wherein R^2 and R^3 are selected from the group consisting of alkyl; $-(CHOH)_n(CH_2)_nOH$, wherein n is 0, 1, 2, 3, 4, 5, or 6; $-(CH_2)_nCO_2R^1$, $-(CH_2)_nCO_2R^4$; hydroxy(C_{1-6})alkyl-; alkenyl; alkyl(CO_2H), alkenyl(CO_2H), alkynyl(CO_2H), or aryl, or R^2 and R^3 can together constitute a bridge of the formula $-(CH_2)_m-$, wherein m is 3, 4, 5, or 6, and wherein R^4 is selected from the group consisting of aryl, alkaryl, or aralkyl, including acetyl, propionyl, and butyryl.

26. The method of claim 17, wherein B is a heterocyclic or alkylheterocyclic group.

27. The method of claim 26, wherein the heterocycle is partially or totally hydrogenated.

28. The method of claim 17, wherein B is the residue of a pharmaceutically-active compound or drug which is directly linked to A-SC(S)- or linked through a divalent linking moiety.

29. The method of claim 17, wherein B is selected from the group consisting of probucol, nicotinic acid, aspirin, coumadin, verapamil, diltiazem, nifedipine, captopril, enalapril, propranolol, terbutalol, labetalol, ibuprofen,

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indomethacin, fenoprofen, mefenamic acid, flufenamic acid, sulindac, and a corticosteroid.

30. The method of claim 17, wherein the dithiocarbamate is an amino acid derivative of the structure $\text{AO}_2\text{C-R}^9\text{-NR}^{10}\text{-C(S)SA}$, wherein R_9 is B or the internal residue of an amino acid and R^{10} is hydrogen or lower alkyl.

31. The method of claim 17, wherein B is a polymer to which one or more dithiocarbamate groups are attached, either directly, or through any suitable linking moiety.

32. The method of claim 17, wherein the polymer is biodegradable.

33. The method of claim 32, wherein the polymer is selected from the group consisting of peptides, proteins, nucleoproteins, lipoproteins, glycoproteins, synthetic and natural polypeptides and polyamino acids, polyorthoesters, poly(α -hydroxy acids), polyanhydrides, polysaccharides, and polycaprolactone.

34. The method of claim 1, wherein B-C(S)S- is pyrrolidine-N-carbodithioate.

35. The method of claim 17 wherein the cardiovascular disease is atherosclerosis.

36. The method of claim 17, wherein the cardiovascular disease is post-angioplasty restenosis.

37. The method of claim 17, wherein the cardiovascular disease is coronary artery disease.

38. The method of claim 17, wherein the cardiovascular disease is angina.

39. The method of claim 17, wherein the cardiovascular disease is a small vessel disease.

40. The method of claim 17, wherein the dithiocarbamate is administered in a dosage of between 0.5 and 500 mg/kg body weight.

41. The method of claim 17, wherein the dithiocarbamate is administered by perfusion balloon catheter.

42. The method of claim 17, wherein the dithiocarbamate is administered in combination with a pharmaceutical agent selected from the group consisting of a lipid lowering agent, a platelet aggregation inhibitor, an antithrombotic agent, a calcium channel blocker, an angiotensin converting enzyme (ACE) inhibitor, a β -blocker, a nonsteroidal antiinflammatory, and a corticosteroid.

43. A method for the suppression of VCAM-1 expression in human cells comprising administering an effective amount of the dithiocarbamate described in claim 17.

44. A method for the treatment of an inflammatory skin disease that is mediated by VCAM-1 comprising administering an effective amount of the dithiocarbamate described in claim 17.

45. A method for the treatment of a human endothelial disorder that is mediated by VCAM-1 comprising administering an effective amount of the dithiocarbamate described in claim 17.

46. The method of claim 45, wherein the disorder is selected from the group consisting of asthma, psoriasis, eczematous dermatitis, Kaposi's sarcoma, multiple sclerosis, and proliferative disorders of smooth muscle cells.

47. A method for the treatment of an inflammatory condition that is mediated by a mononuclear leucocyte comprising administering an effective amount of the dithiocarbamate described in claim 17.

48. The dithiocarbamate disclosed in any of claims 17-34.

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49. A pharmaceutical composition comprising an effective amount to treat cardiovascular disease of a compound disclosed in any of claims 17-34.

50. A pharmaceutical composition comprising an effective amount to treat a disorder mediated by VCAM-1 of a compound disclosed in any of claims 17-34.

Effects of Linoleic Acid and 13-HPODE on the Kinetics of VCAM-1 Gene Expression

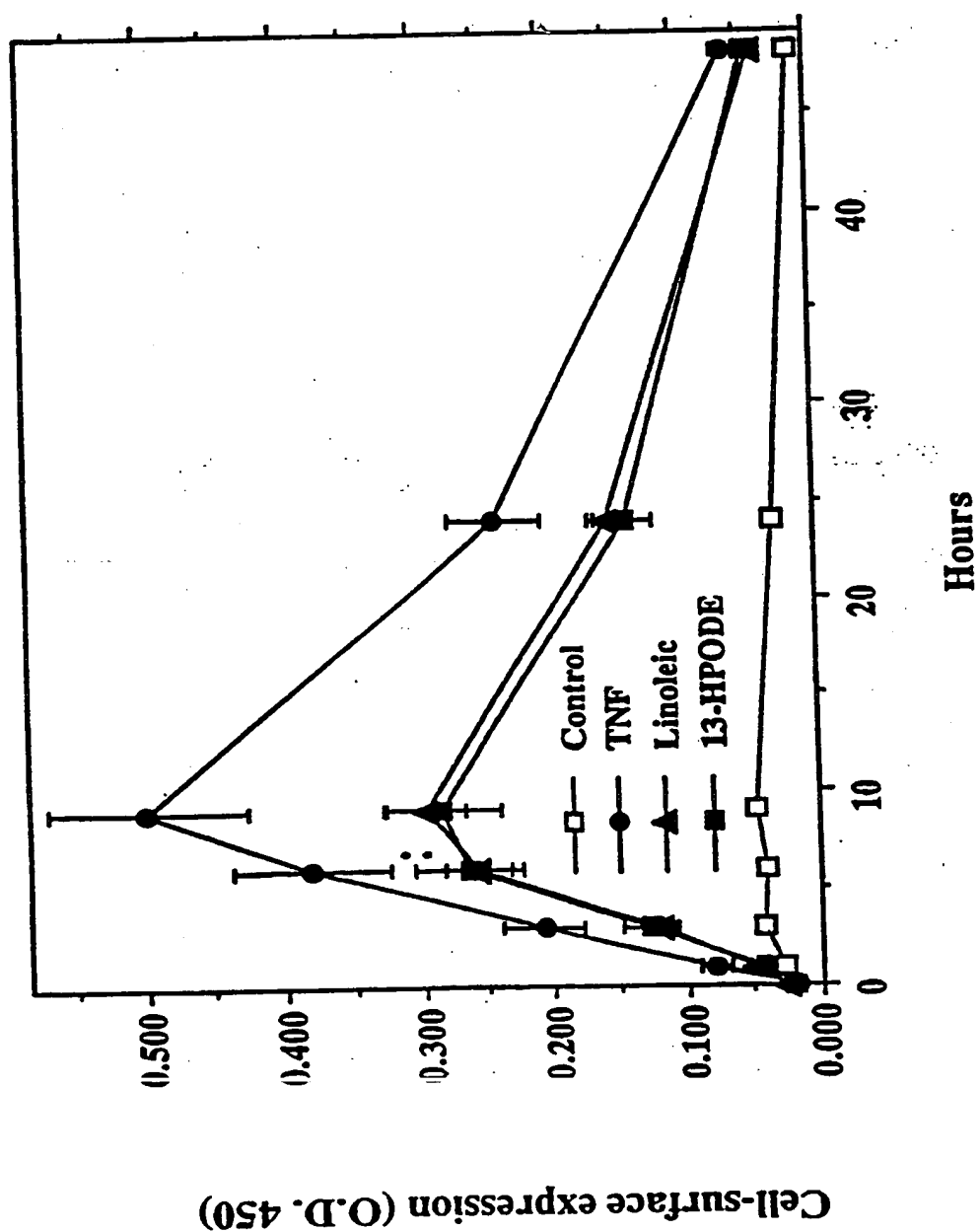


Figure 1

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Effect of Linoleic Acid and 13-HPODE Dose Response on VCAM-1 Gene Expression

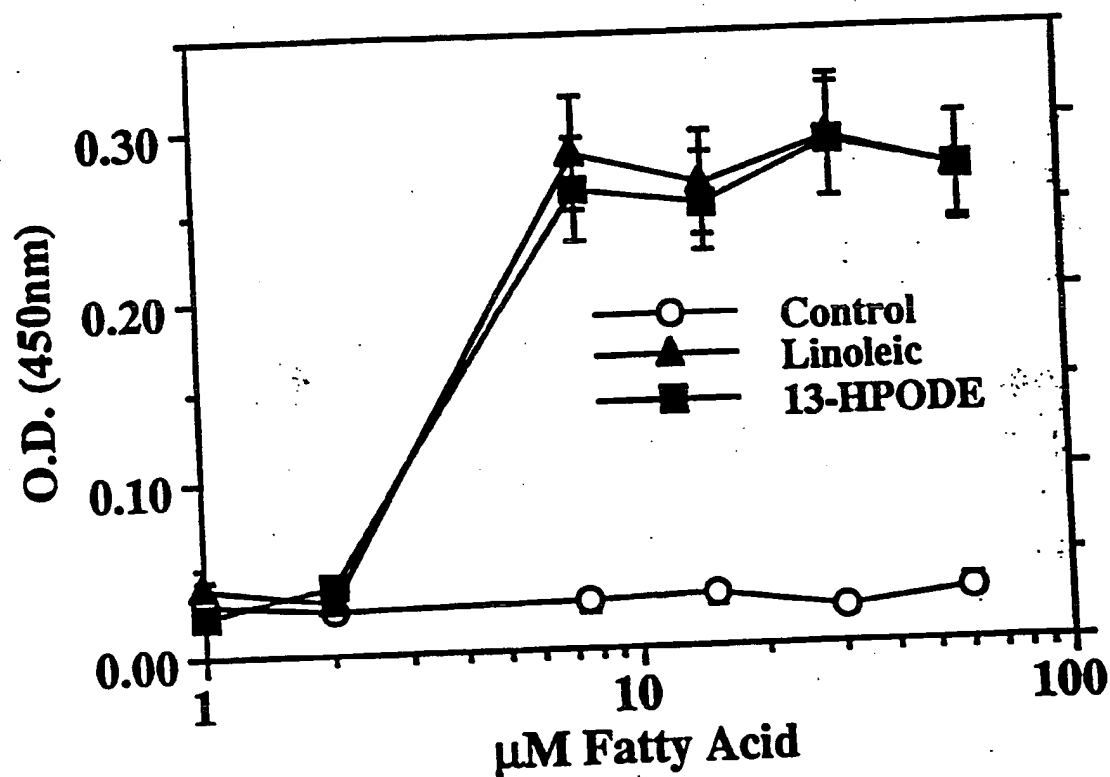


Figure 2

3 / 2 0

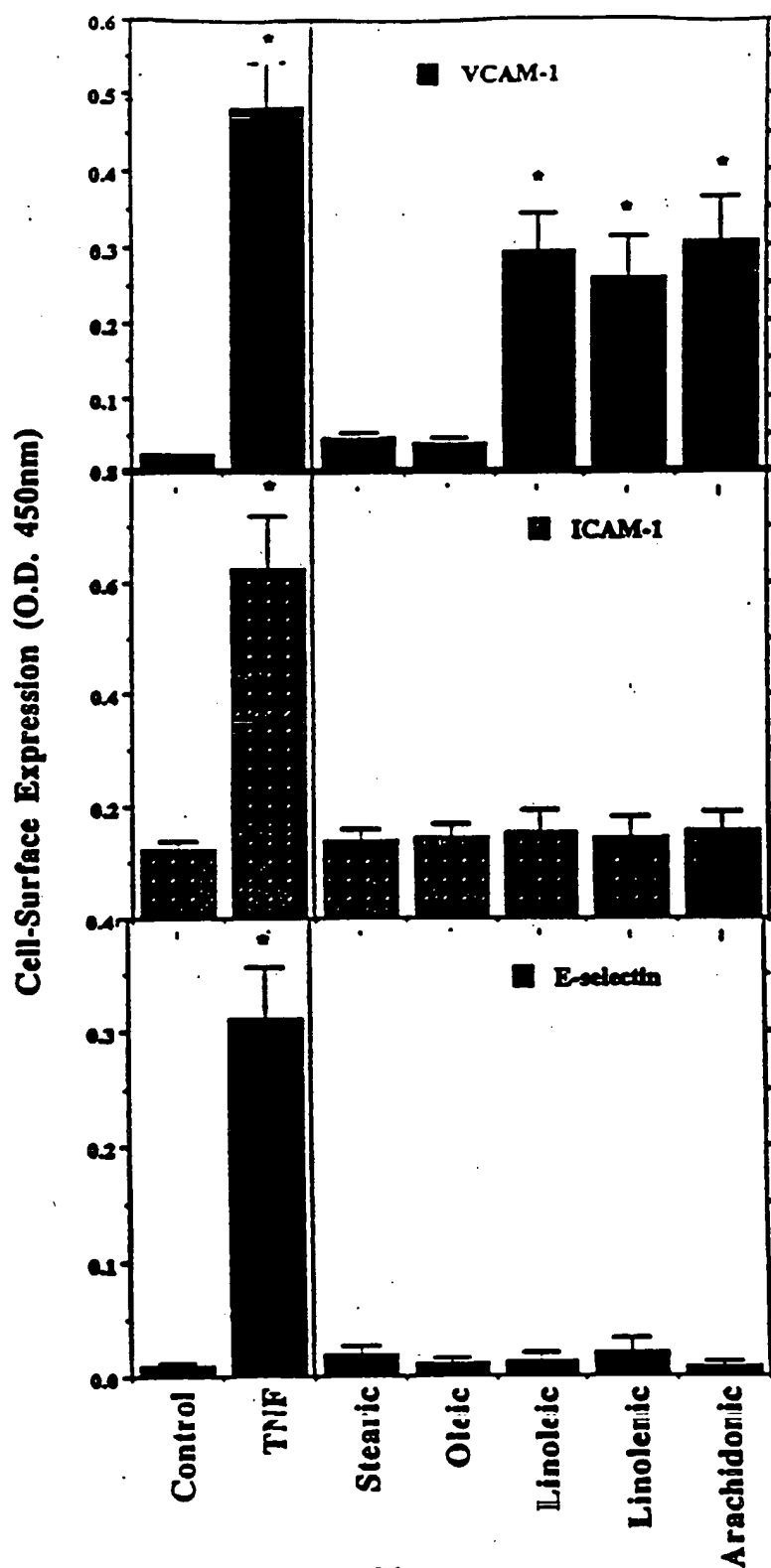


Figure 3

4 / 2 0

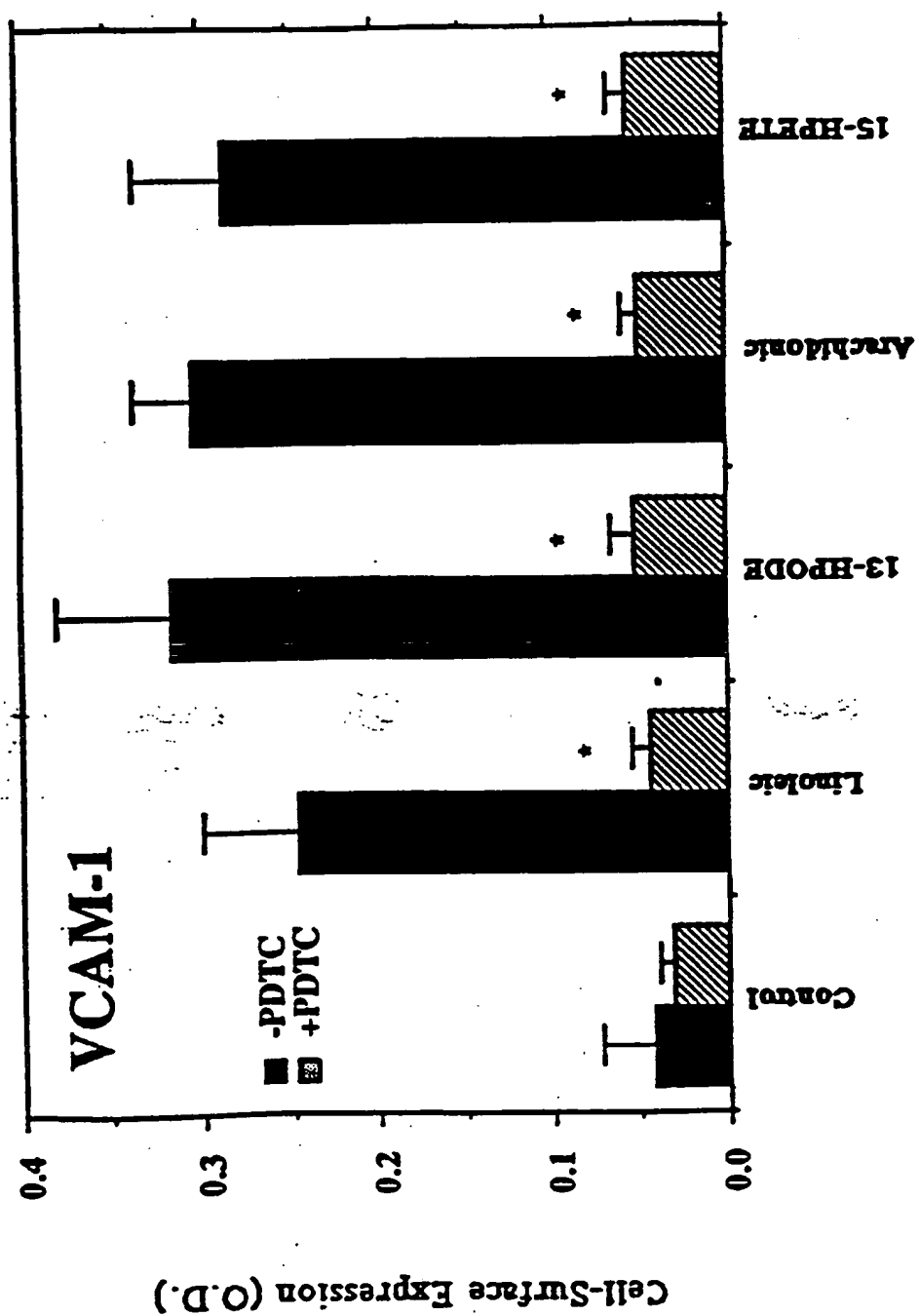


Figure 4

5 / 2 0

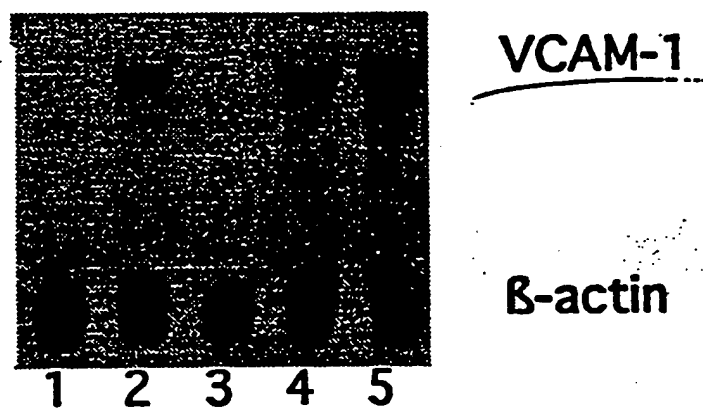
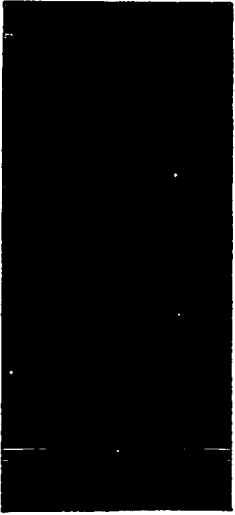


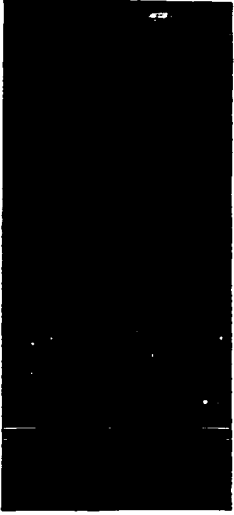
Figure 5

6 / 2 0

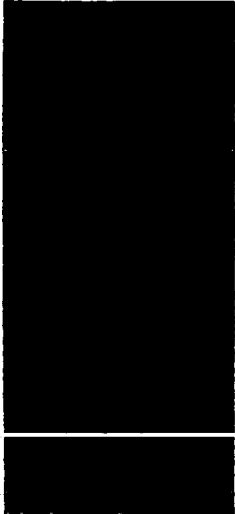
A.A. + CHX
A.A.
L.A.
L.A. + CHX
CHX
CON



A.A. + CHX
A.A.
L.A.
L.A. + CHX
CHX
CON



A.A. + CHX
A.A.
L.A.
L.A. + CHX
CHX
CON



A.A. + CHX
A.A.
L.A.
L.A. + CHX
CHX
CON




Figure 6

7 / 2 0

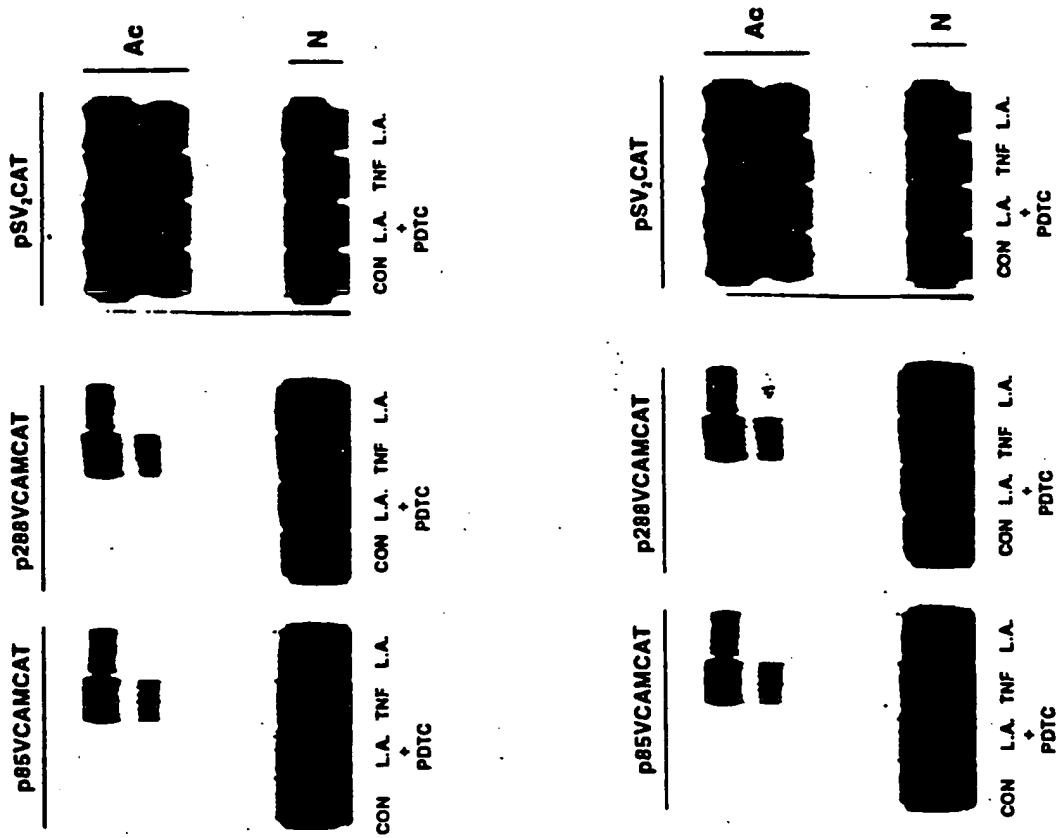


Figure 7

8 / 2 0

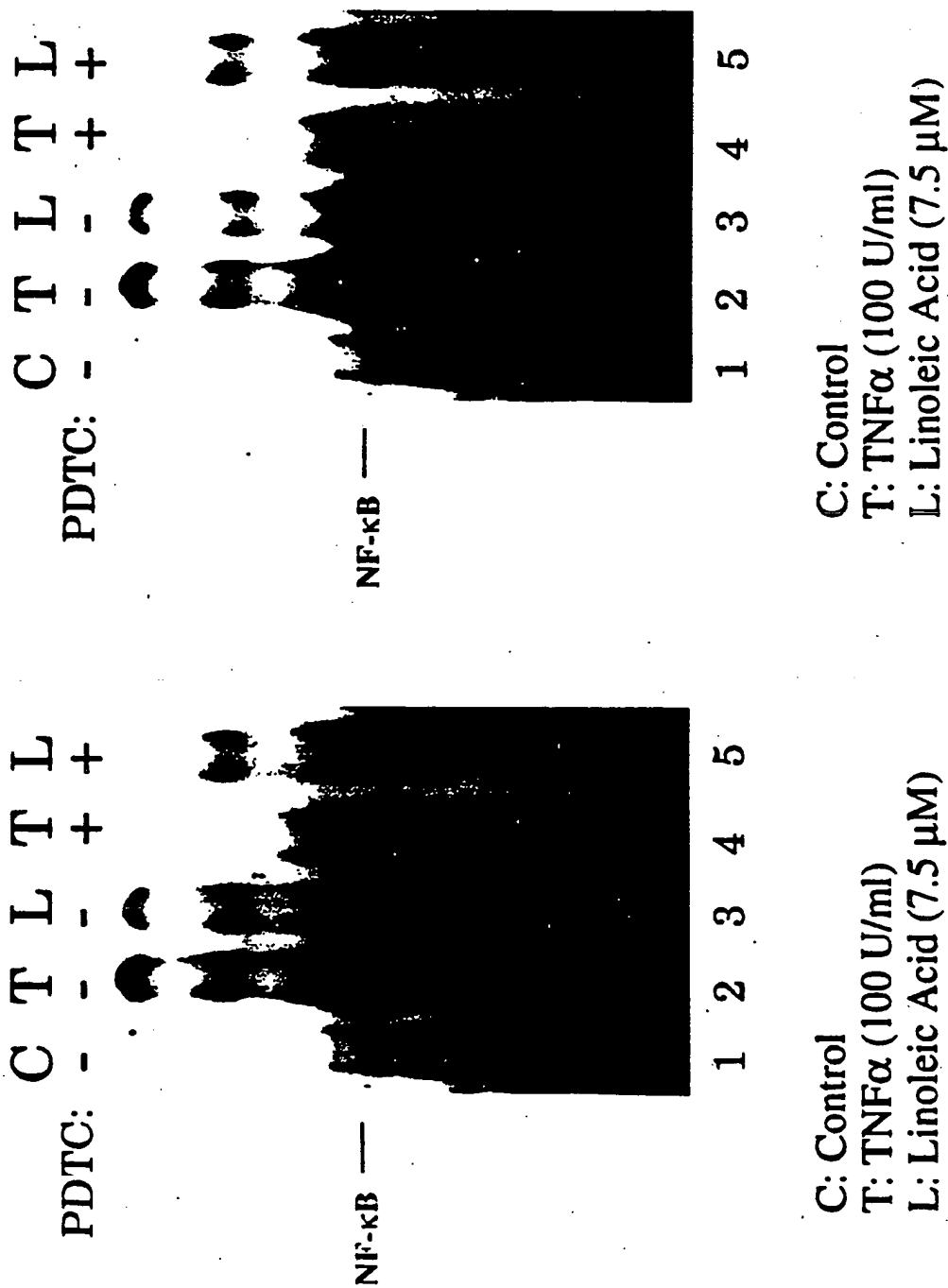
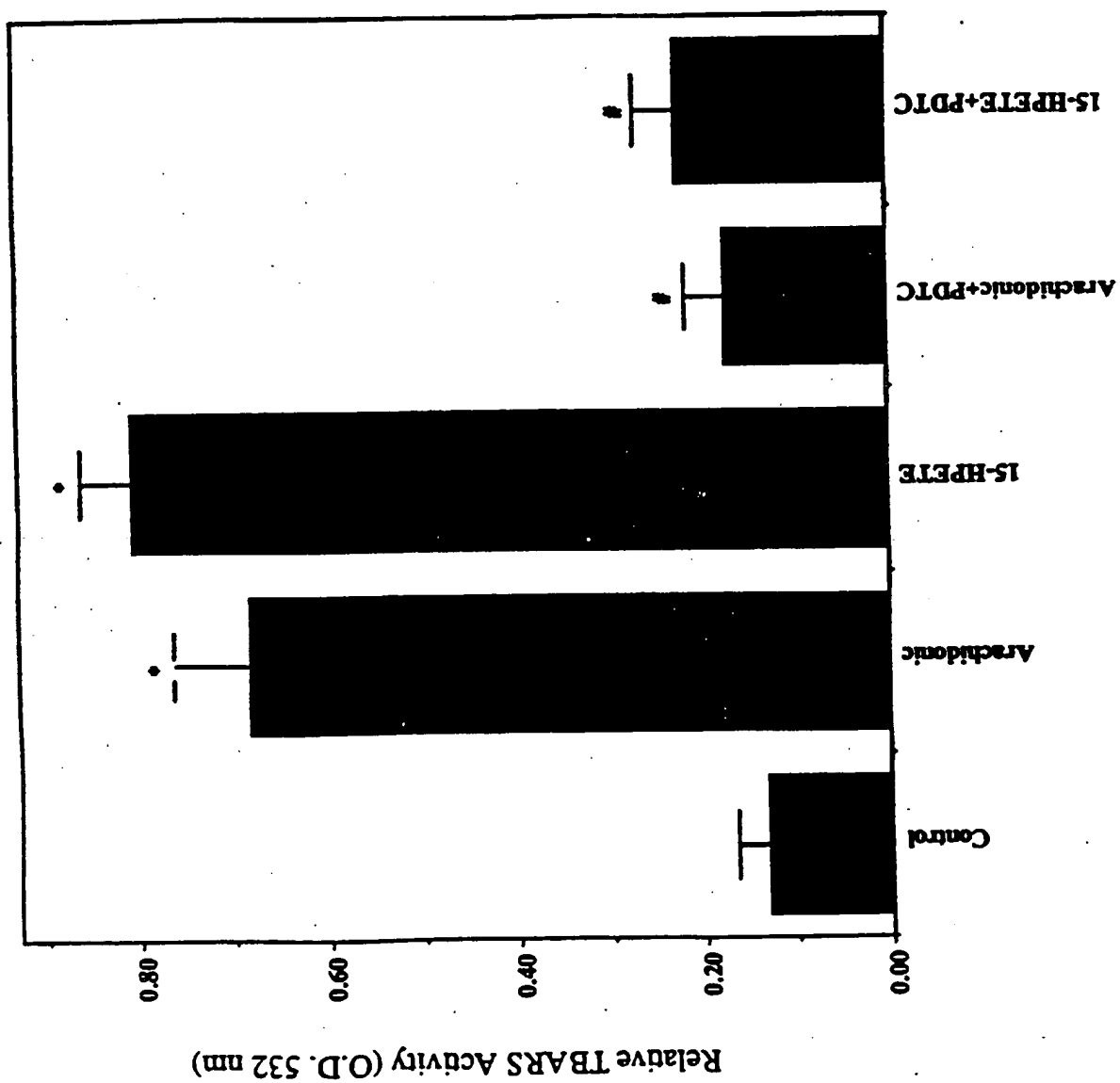


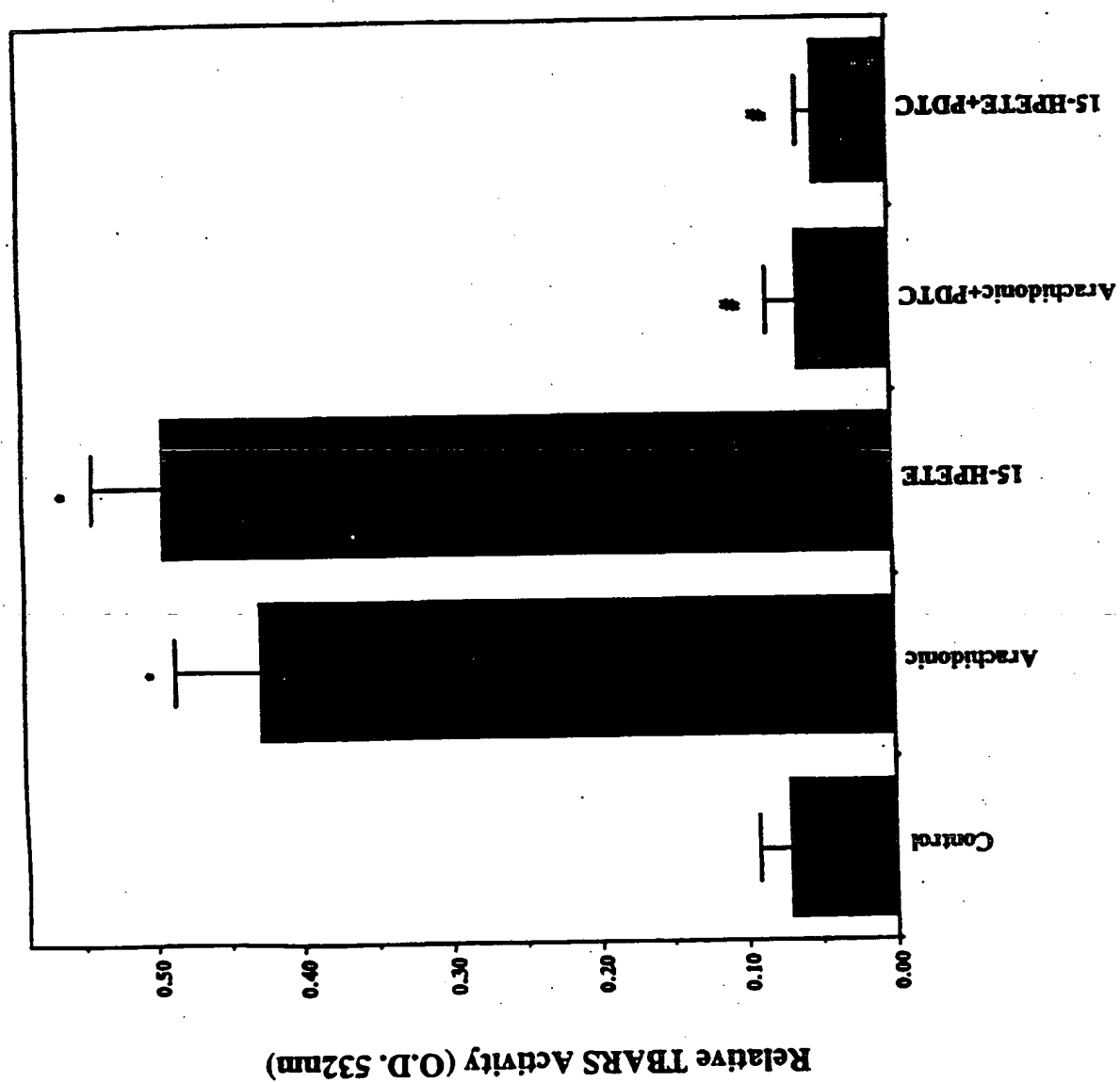
Figure 8

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*-value differs ($p < 0.05$) from Control
#-value differs ($p < 0.05$) from respective fatty acid not treated with PDTC

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*-value differs ($p < 0.05$) from Control
*-value differs ($p < 0.05$) from respective fatty acid not treated with PDTC

Figure 9b

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Figure 10a

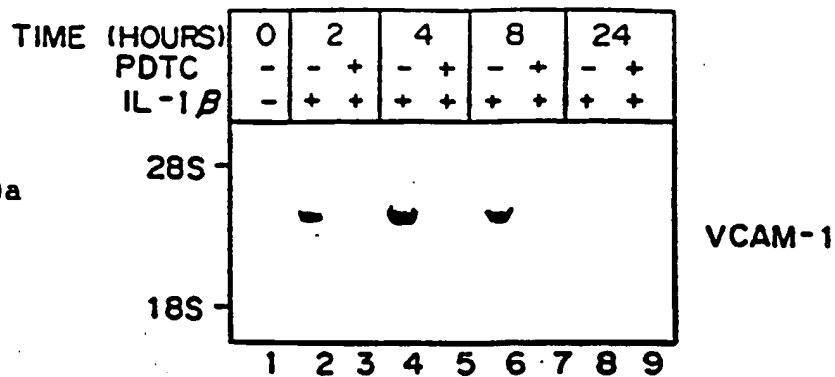


Figure 10b

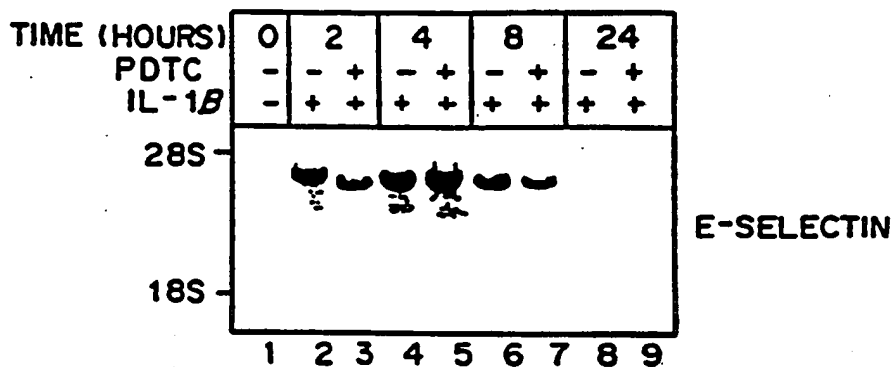


Figure 10c

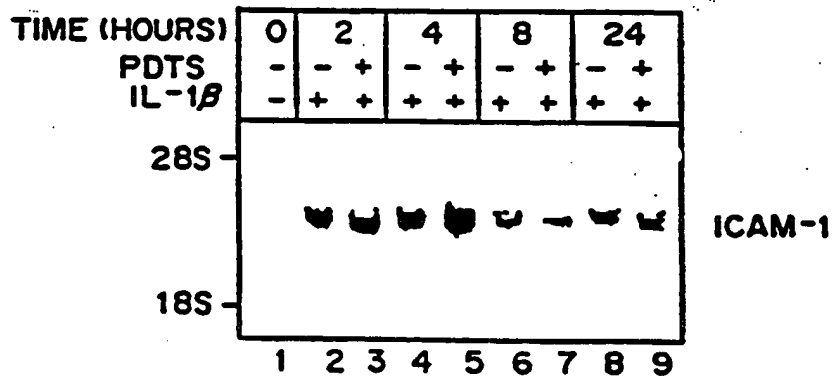
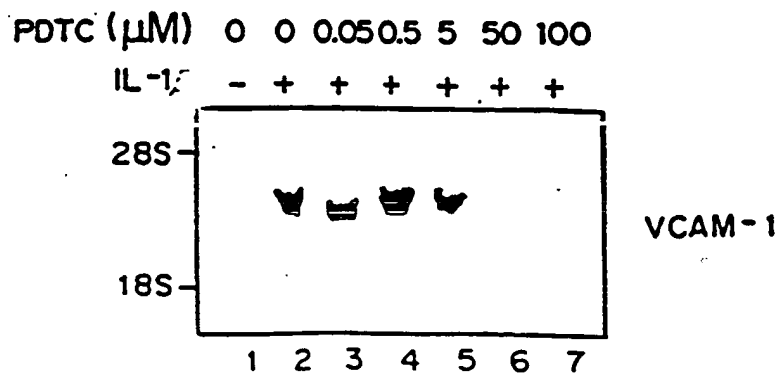
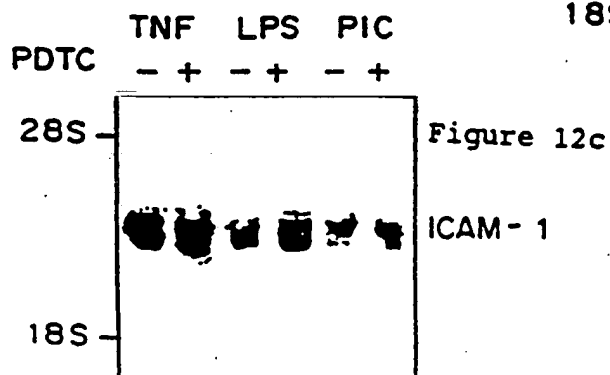
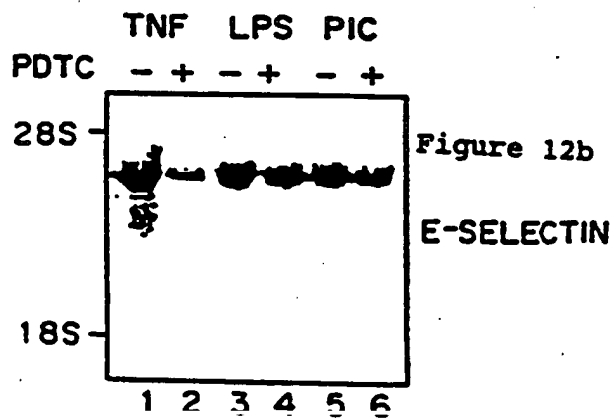
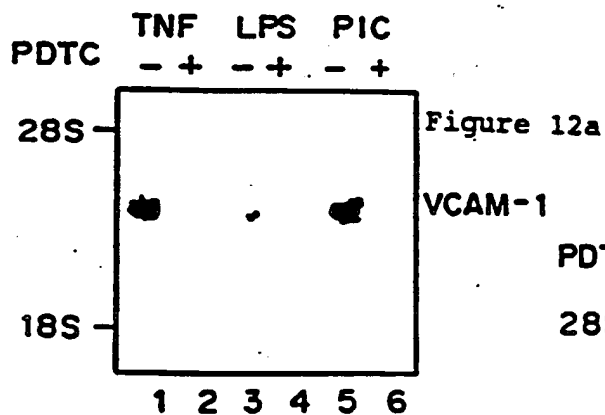
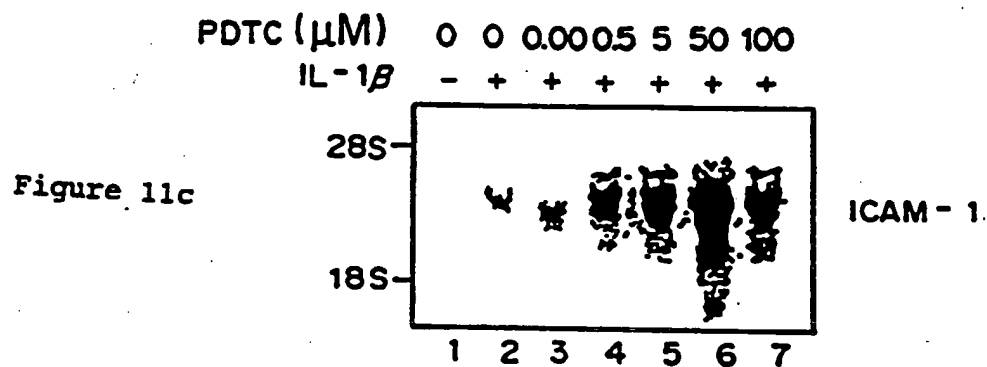
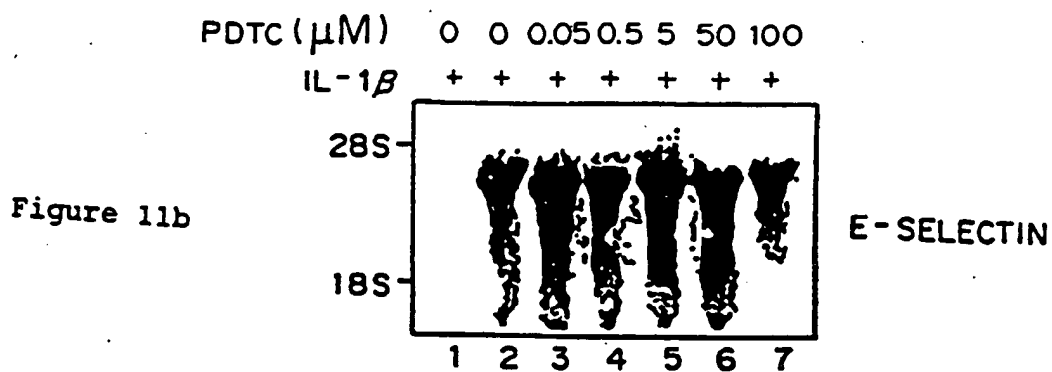


Figure 11a



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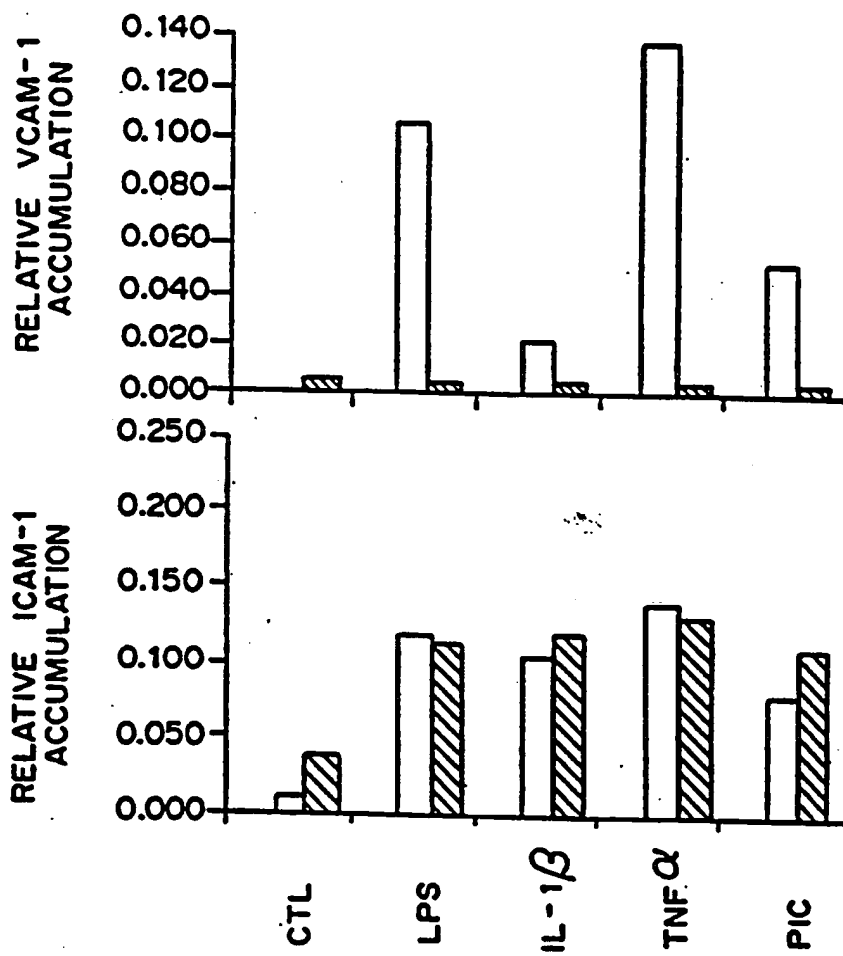


Figure 13

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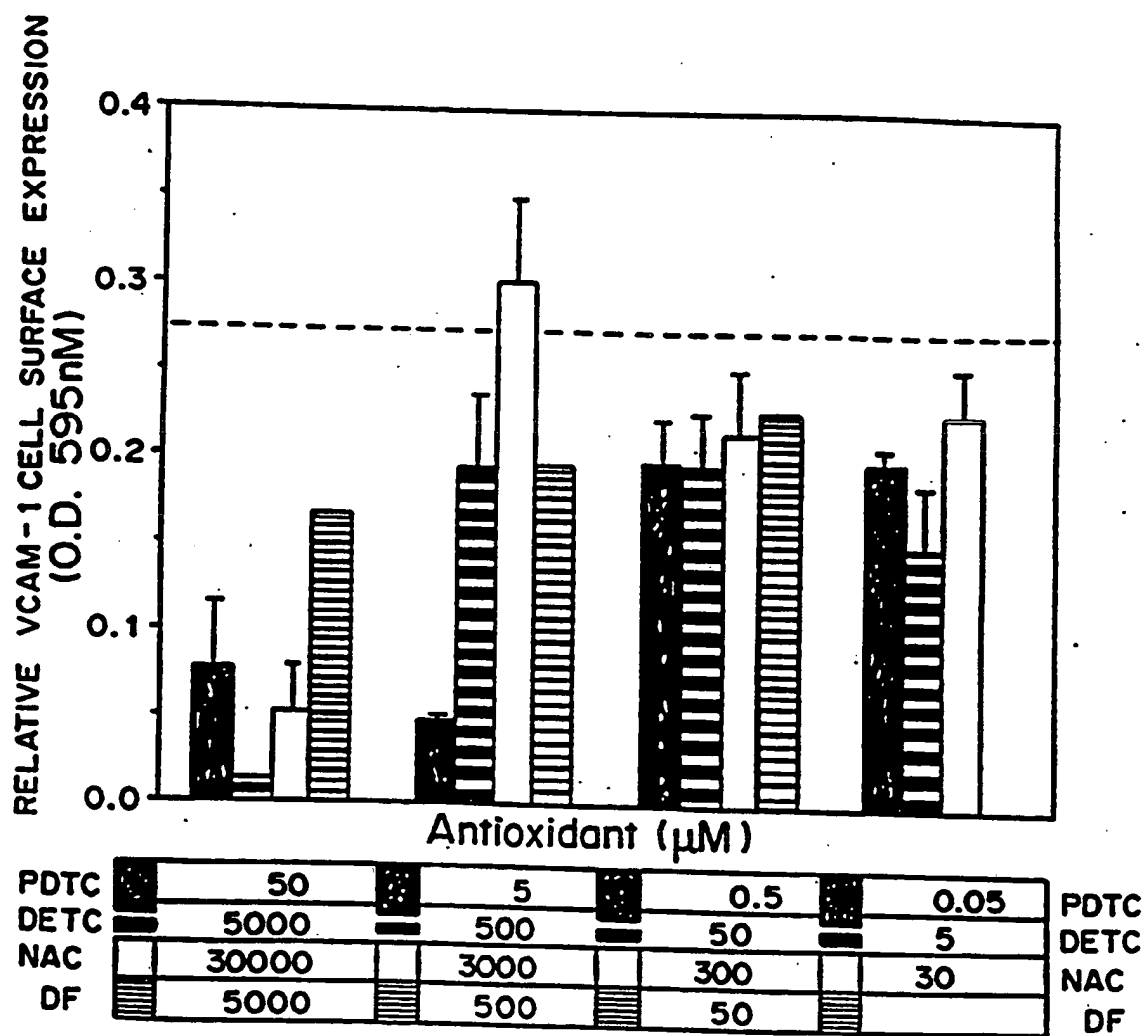


Figure 14

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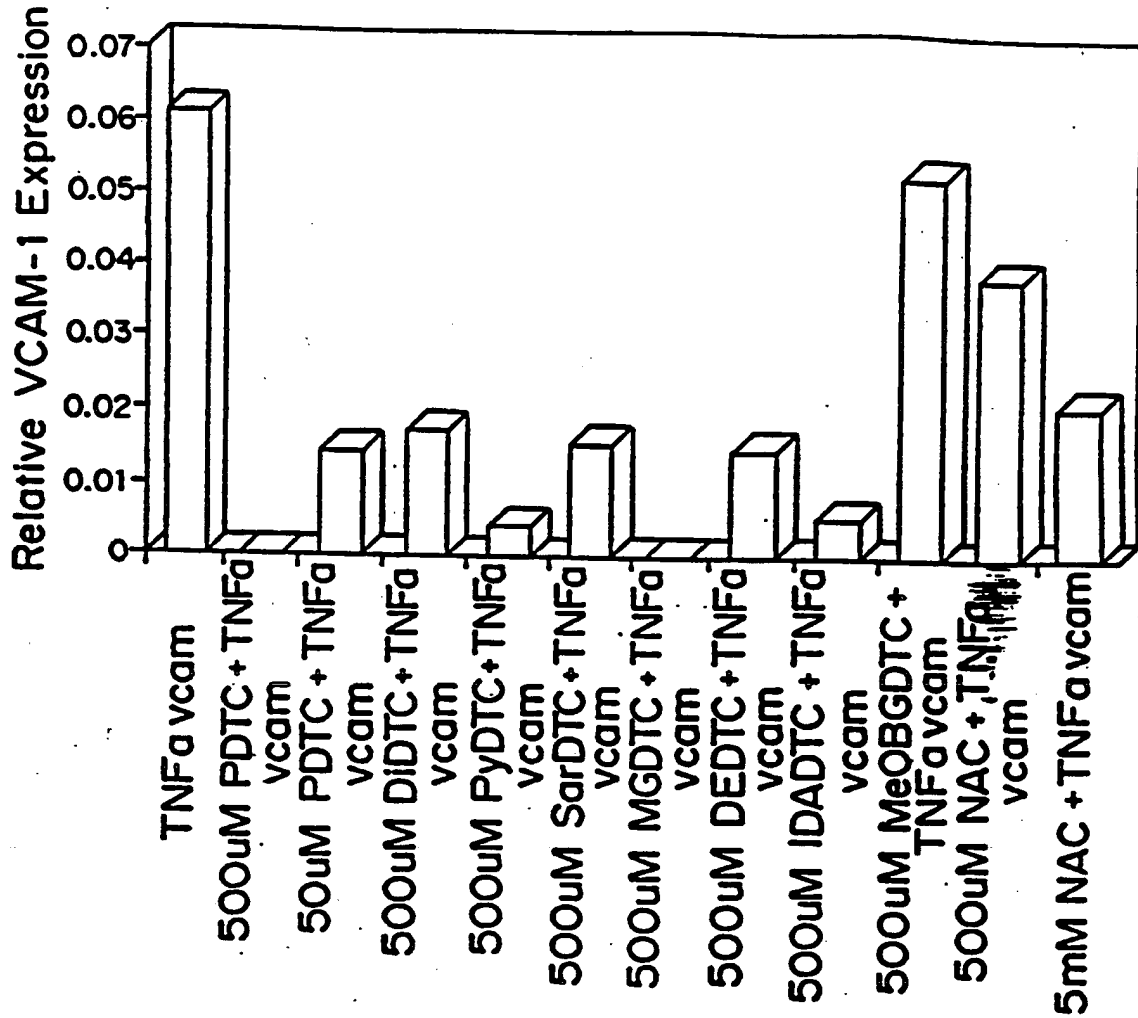
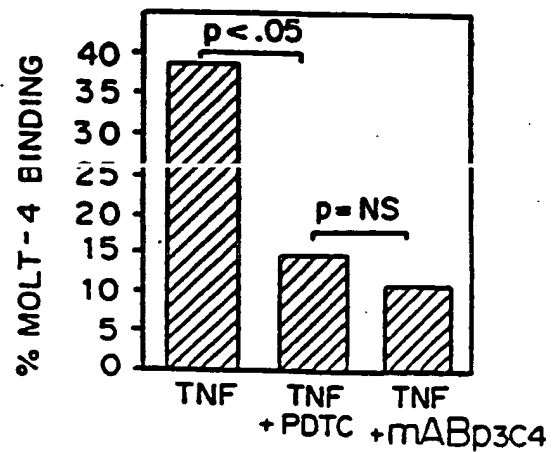
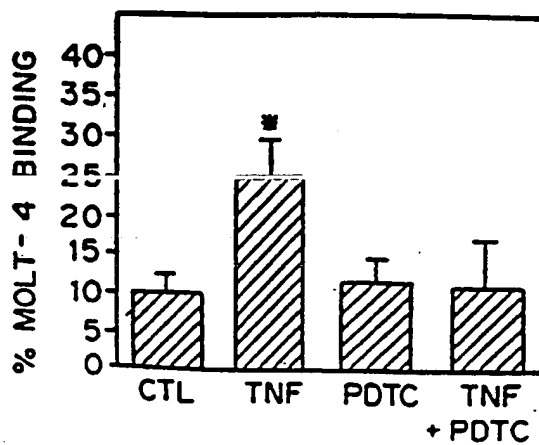
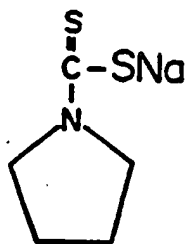
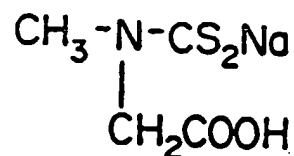
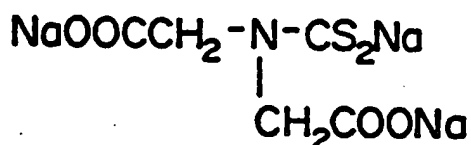
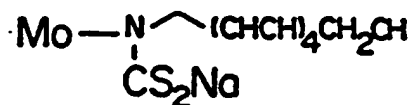
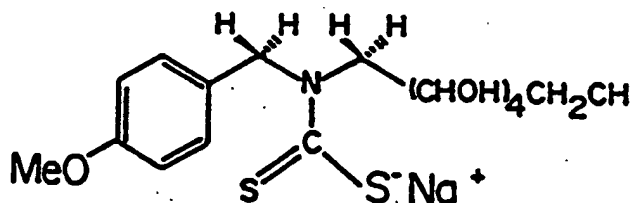
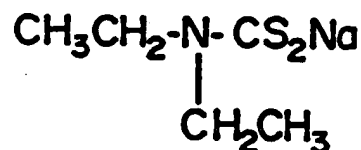
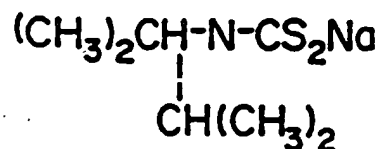


Figure 15



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Figure 17

SODIUM PYRROLIDINE-*N*-CARBODITHIOATESODIUM *N*-METHYL-*N*-CARBOXYMETHYL-*N*-CARBODITHIOATE
(OR SODIUM SARCOSINEDITHIOCARBAMATE)TRISODIUM *N,N*-di(CARBOXYMETHYL)-*N*-CARBODITHIOATE
(OR IMINODIACETIC ACID DITHIOCARBAMATE, TRISODIUM)SODIUM *N*-METHYL-D-GLUCAMINE-*N*-CARBODITHIOATESODIUM *N*-(4-METHOXYBENZYL)-D-GLUCAMINE-*N*-CARBODITHIOATESODIUM *N,N*-DIETHYL-*N*-CARBODITHIOATE (OR SODIUM DIETHYLDITHIOCARBAMATE)SODIUM *N,N*-DIISOPROPYL-*N*-CARBODITHIOATE
(OR SODIUM DIISOPROPYLDITHIOCARBAMATE)

Effect of PDTC on the formation of fluorescent adducts

(1 Micromol of 13-HPODE was incubated with 200 micrograms of BSA in the presence of PDTC for 6 days. Fluorescence was measured at 430-460 nm with excitation set at 330-360 nm)

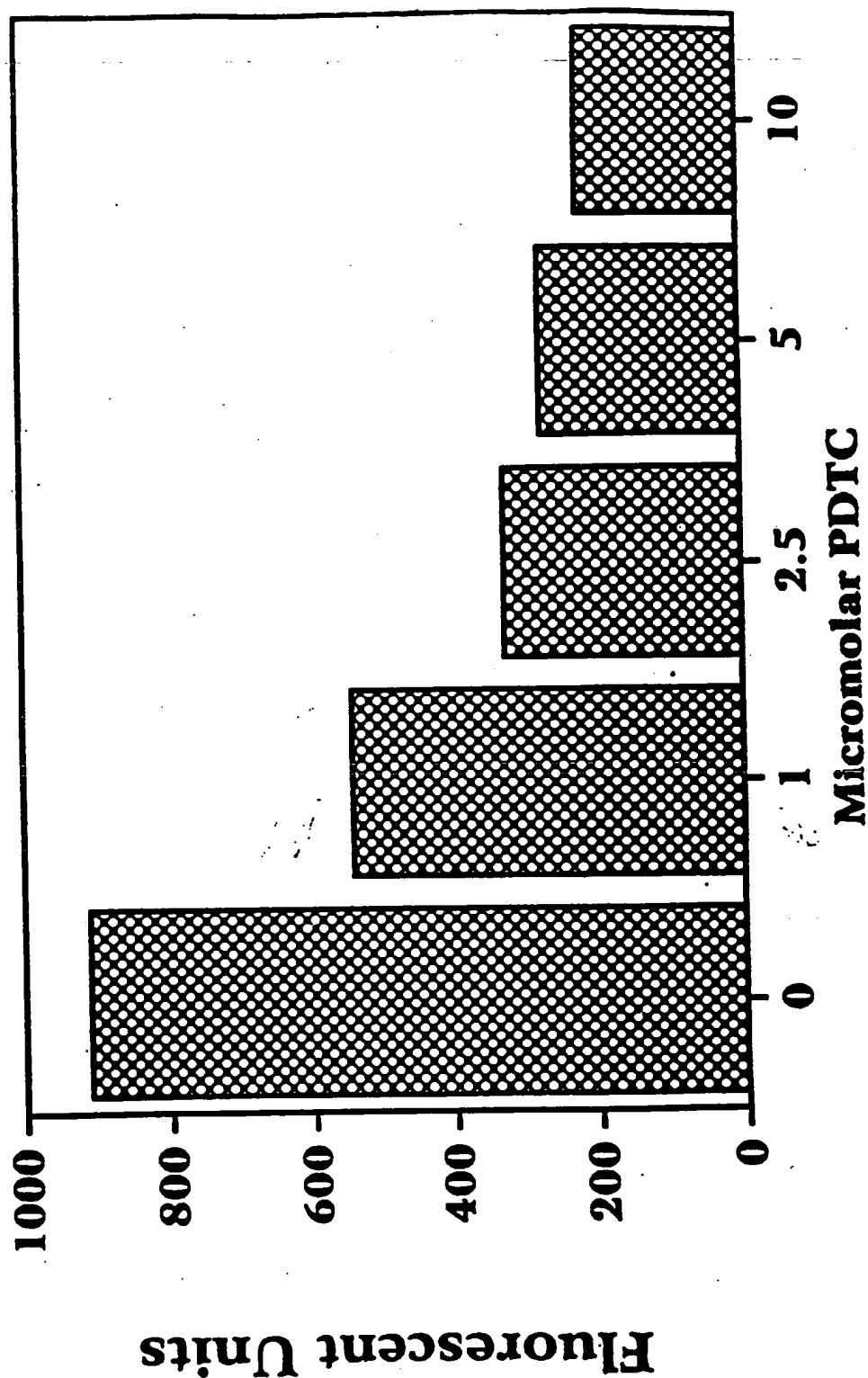


Figure 18

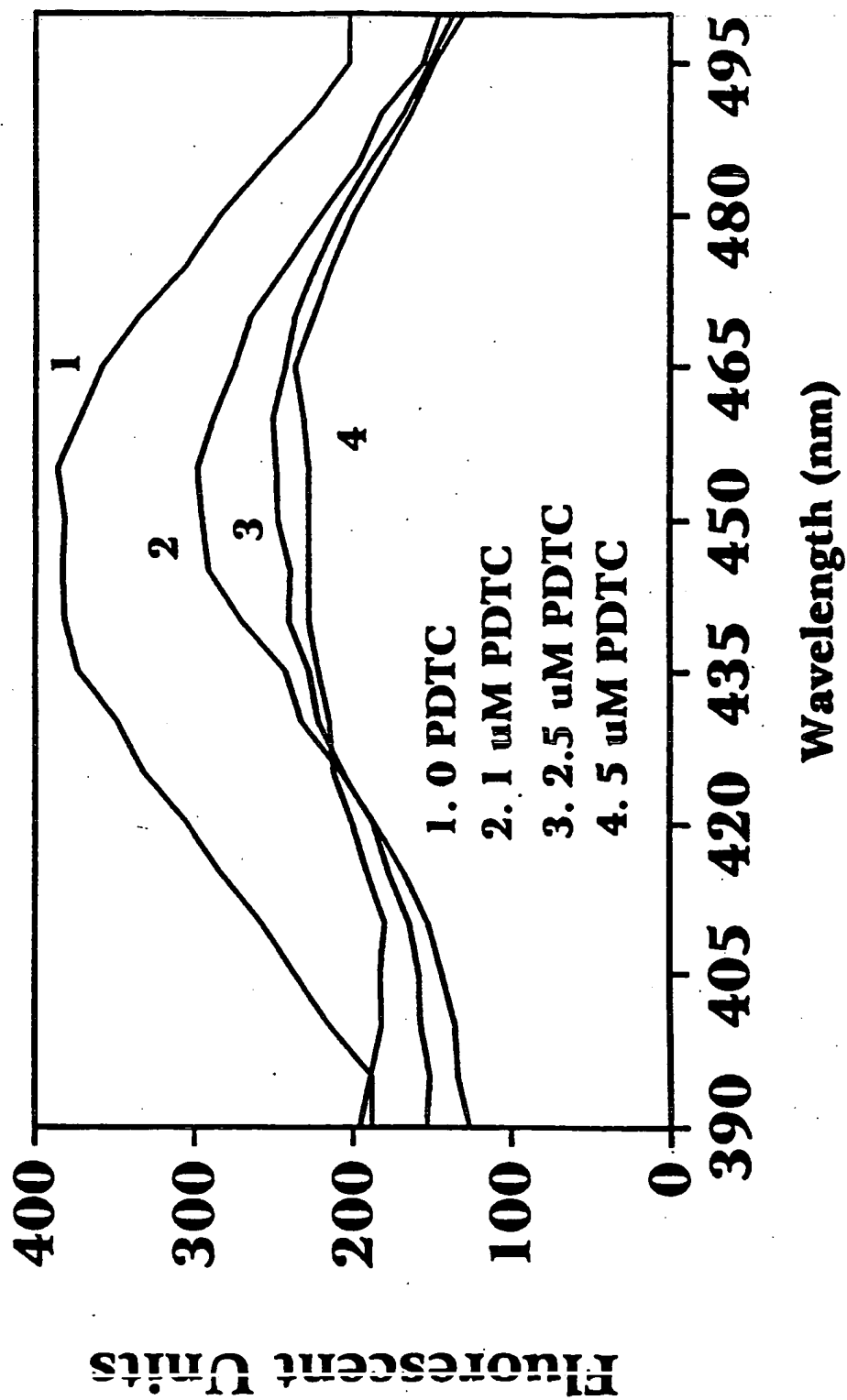
Effect of PDTC on the formation of fluorescent adducts from BSA and LOOH

Figure 19

Effect of PDTC on the Oxidation of LDL by HRP

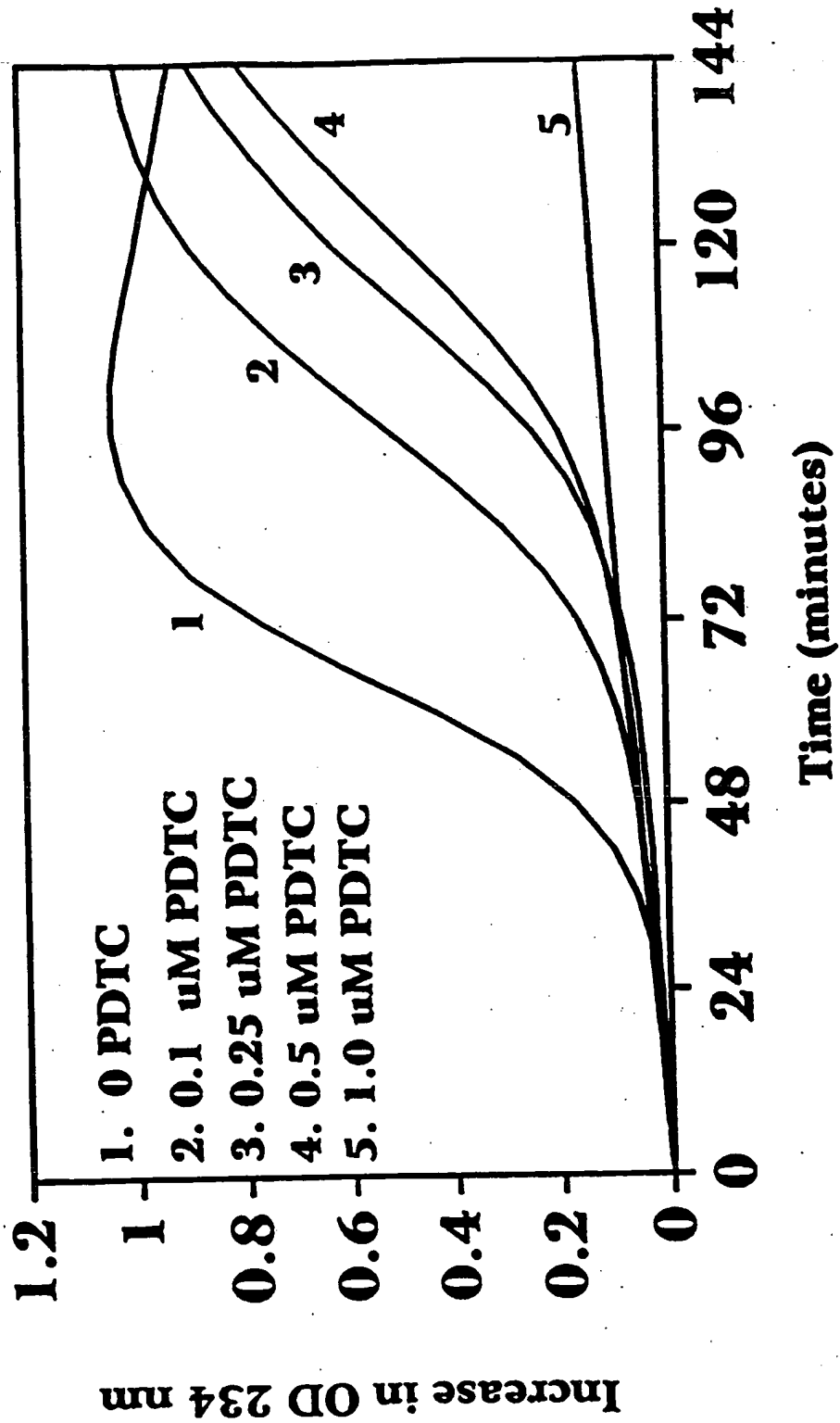


Figure 20

Cells	Linoleic	Ox-linoleic
Control	9561	11445
TNF α	8874	27894
IL-1 β	9134	25813*
TNF α +PDTC	8444	7966#
IL-1 β +PDTC	8257	8012#

*-value differs ($p < 0.05$) from Control

#-value differs ($p < 0.05$) from corresponding group not treated with PDTC

Figure 21

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US95/05880

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US. CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LABORATORY INVESTIGATION, Volume 64, No. 3, issued 1991, Poher et al., "What Can Be Learned From the Expression of Endothelial Adhesion Molecules in Tissues?," pages 301-305, see entire document.	1-6, 11-13, 43-50
X	INTERNATIONAL JOURNAL OF IMMUNOPHARMACOLOGY, Volume 6, No. 3, issued 1984, Corke et al., "The Influence of Diethyl-dithiocarbamate ('Imuthiol') on Mononuclear Cells in Vitro," pages 245-247, see entire document.	48-50
Y		1-6, 1-13, 43-50
X	US, A, 4,870,101 (KU ET AL.) 26 September 1989, see entire document.	48-50
Y		1-6, 11-13, 43-50

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be part of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 SEPTEMBER 1995

Date of mailing of the international search report

13 SEP 1995

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 PETER G. O'SULLIVAN

Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05880

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, 119:23788, issued 1993, Pasqualini et al., "Brain-tropic Radiopharmaceutical Compounds Comprising a Transition Metal Nitride Complex, and Preparation Method Therefor," see abstract No. 23788, WO 9301839.	48-50
Y	US, A, 4,670,471 (CLARK) 02 June 1987, see abstract.	1-6, 11-13, 43-50
A	THE NEW ENGLAND JOURNAL OF MEDICINE, Volume 320, Number 14, issued 1989, Steinberg et al., "Beyond Cholesterol: Modifications of Low-Density Lipoprotein That Increase Its Atherogenicity, pages 915-924.	1-50
X	Chemical Abstracts, Volume 81, issued 1974, Tiwari et al., "Possible Anti-Parkinsonian Compounds IV. Synthesis of	48-50
Y	Piperazine bis[amino(thiocarbonyl)thio-acetates and propionates]," see page 527, column 1, abstract No. 105447b.	48-50
A	JOURNAL OF CLINICAL INVESTIGATION, Volume 77, No. 2, issued 1986, Parthasarathy et al., "Probucol Inhibits Exudative Modification of Low Density Lipoprotein," pages 641-644.	1-50
X	THE LANCET, Volume II, No. 8613, issued 1988, Lang et al., "Randomized, Double-Blind, Placebo-Controlled Trial of	48-50
Y	Dithiocarb Sodium ('Imuthiol') In Human Immunodeficiency Virus Infection," pages 702-706, see entire document.	48-50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05880

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05880

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 31/21, 31/40, 38/06; C07C 327/00, 327/36, 333/16, 333/20, 333/24, 333/32; C07D 207/04; C07K 5/09

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

514/18,423, 478, 479, 484, 485, 487, 488, 489, 506, 513, 824, 825, 826, 861, 863, 886; 530/331; 548/431; 558/230, 235; 564/76; 568/21, 25

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

514/18,423, 478, 479, 484, 485, 487, 488, 489, 506, 513, 824, 825, 826, 861, 863, 886; 530/331; 548/431; 558/230, 235; 564/76; 568/21, 25

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-50, drawn to heterocyclic dithiocarbamate compounds, compositions and methods.
- II. Claims 1-6, 8-25, 28-30, 35-50, drawn to non-polymeric, non-heterocyclic dithiocarbamate compounds, compositions and methods.
- III. Claims 1-6, 8-25, 28-30, 35-50, drawn to non-polymeric, non-heterocyclic thiuram disulfide compounds, compositions and methods.
- IV. Claims 1-6, 8-24, 31-33, 35-50, drawn to polymeric dithiocarbamate compounds, compositions and methods.
- V. Claims 1-6, 9-17, 23, 30, 35-50, drawn to dithiocarboxylate ester compounds, compositions and methods.
- VI. Claims 1-6, 8-17, 23, 25, 28, 35-50, drawn to bithiocarbonylsulfide compounds, compositions and methods.

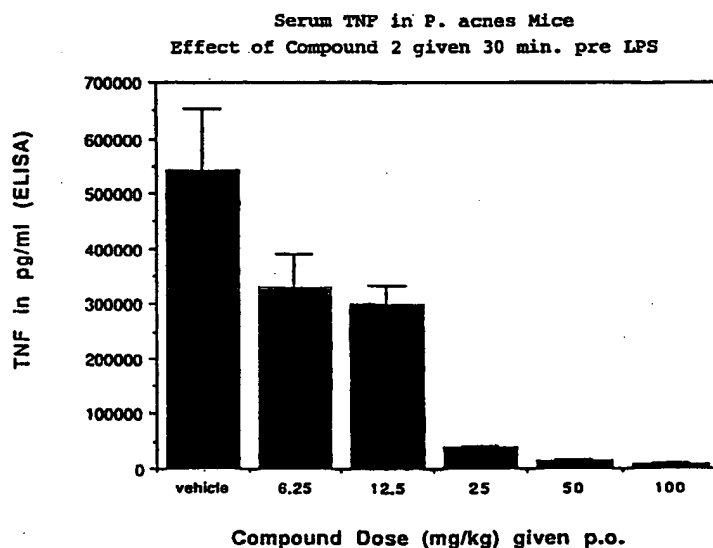
The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the active compounds of each group are structurally diverse from the other groups so that references for one group would not necessarily suggest the others to one skilled in the art.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A01N 43/06, 43/40, A61K 31/415		(11) International Publication Number: WO 90/15534
A1		(43) International Publication Date: 27 December 1990 (27.12.90)
<p>(21) International Application Number: PCT/US90/03380</p> <p>(22) International Filing Date: 12 June 1990 (12.06.90)</p> <p>(30) Priority data: 365,387 13 June 1989 (13.06.89) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 365,387 (CIP) Filed on 13 June 1989 (13.06.89)</p> <p>(71) Applicant (for all designated States except US): SMITH-KLINE BEECHAM CORPORATION [US/US]; One Franklin Plaza, P.O. Box 7929, Philadelphia, PA 19101 (US).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only) : HANNA, Nabil [US/US]; 539 Sierra #108, Solana Beach, CA 92075 (US).</p> <p>(74) Agents: DINNER, Dara, L. et al.; Corporate Patents - U.S., N160, SmithKline Beecham Corporation, P.O. Box 7929, Philadelphia, PA 19101 (US).</p> <p>(81) Designated States: AU, CA, JP, KR, US.</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>		

(54) Title: MONOKINE ACTIVITY INTERFERENCE



(57) Abstract

A method of treating a human afflicted with a T Cell Viral (TIV) infection, such as a human immunodeficiency virus (HIV) infection, which comprises administering to such human an effective, monokine activity interfering amount of a monokine activity interfering agent.

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DK	Denmark				

5

TITLE

10

MONOKINE ACTIVITY INTERFERENCE

BACKGROUND OF THE INVENTION

15

This invention relates to a method of treating a human afflicted with a human immunodeficiency virus (HIV), which comprises administering to such human an effective amount of a monokine activity interfering agent.

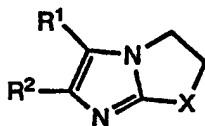
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The human acquired immune deficiency syndrome (AIDS) results from the infection of T lymphocytes with Human Immunodeficiency Virus (HIV). At least three types or strains of HIV have been identified, i.e., HIV-1, HIV-2 and HIV-3. As a consequence of HIV infection, T-cell mediated immunity is impaired and infected individuals manifest severe opportunistic infections and/or unusual neoplasms. There is a continuing need for agents which are useful in inhibiting further disease progress in an already infected individual.

25

Bender *et al.*, U.S. Patent Number 4,794,114, issued December 27, 1988, disclose a method of inhibiting the production of IL-1 by monocytes and/or macrophages in a human in need thereof which comprises administering to such human an effective, interleukin-1 production inhibiting amount of a compound of the formula:

30



wherein:

35

One of R¹ and R² must be 4-pyridyl and the other is selected from monohalosubstituted phenyl wherein said substituent is selected from halo or C₁₋₄ alkoxy;

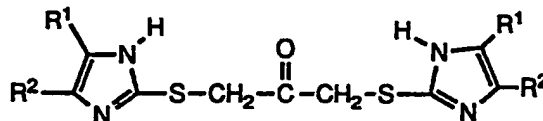
X is CH₂, CH₂CH₂ or S(0)n; and

n is 0, 1 or 2;

or a pharmaceutically acceptable salt thereof.

-2-

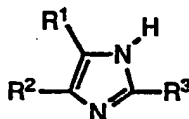
Bender *et al.*, U.S. Patent Number 4,778,806, issued October 18, 1988, claim a method of inhibiting the production of interleukin-1 by monocytes and/or macrophages in a human in need thereof which comprises administering to such human an effective, interleukin-1 production inhibiting amount of a compound of the formula:



wherein:

one of R¹ and R² is 4-pyridyl and the other is monohalosubstituted phenyl; or a pharmaceutically acceptable salt thereof.

Bender *et al.*, U.S. Patent Number 4,780,470, issued October 25, 1988, claim a method of inhibiting the production of interleukin-1 by monocytes and/or macrophages in a human in need thereof which comprises administering to such human an effective, interleukin-1 production inhibiting amount of a compound of the formula:



wherein:

One of R¹ and R² is 4-pyridyl and the other is selected from monohalosubstituted phenyl; and

R³ is S or SCF₂CF₂H;
or a pharmaceutically acceptable salt thereof.

Folks *et al.*, *J. Immunol.*, **136**, 40-49 (1986), discuss that cytokine-induced increase of HIV expression in a chronically infected macrophage cell line was associated with the concomitant and selective increase of IL-1 production.

Koyanagi *et al.*, *Science*, **241**, 1673 (1988), discuss that treatment of primary human mononuclear phagocytes with hematopoietic growth factors including macrophage colony stimulating factor (M-CSF), granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) stimulated HIV production in these cells.

Clouse *et al.*, *J. Immunol.*, **142**, 431 (1989), discuss that monokines secreted by activated human monocytes induced elevated levels of HIV expression in a chronically infected human T cell clone. The monokine involved in this process was identified as TNF α .

Gowda *et al.*, *J. Immunol.*, **142**, 773 (1989), discuss that T cell activation is required for HIV entry and HIV-dependent cell fusion.

Zagury et al., Science, 231, 850 (1986), discuss that T cell activation is required for HIV gene expression.

5 Wright et al., J. Immunol., 141, 99 (1988), discuss that monocytes from HIV-infected patients produced large amounts of TNFa and IL-1 upon culturing in vitro.

Ensoli et al., Science, 243, 223 (1989), discuss that IL-1 is produced by cells from Kaposi's sarcoma lesions of HIV-infected patients, and that antibodies to IL-1 inhibited cell proliferation in vitro.

10 Beutler et al., Nature (London), 316, 552-554 (1985), discuss the role of TNFa in cachexia.

Beutler et al., J. Immunol., 135, 3969-3971 (1985), discuss the role of IL-1 in cachexia.

Baracos et al., N. Eng. J. Med., 308, 553-558 (1983), discuss the role of IL-1 in muscle degeneration.

15 Chiebowski et al., Nutr. Cancer, 7, 85 (1985), discuss HIV-associated states of cachexia and muscle degradation.

Lahdevirta et al., The American J. Med., 85, 289 (1988), discuss that TNFa is involved in the HIV-associated states of cachexia and muscle degradation.

20 Wright et al., J. Immunol. 141(1):99-104 (1988) suggests a possible role for TNF in AIDS cachexia by elevated serum TNF and high levels of spontaneous TNF production in peripheral blood monocytes from patients.

25 Lee et al., Int. J. Immunopharmac., 10(7), 835 (1988), discuss that [5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo(2,1-B)thiazole], which inhibits both 5-lipoxygenase and cyclooxygenase-mediated arachidonate metabolism, was shown to be a potent inhibitor of IL-1 production by bacterial lipopolysaccharide (LPS)-stimulated human monocytes.

30 Badger et al., Circulatory Shock, 27(1), 51-61 (1989), discuss the protection by [5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo(2,1-B)thiazole] against two mouse models of LPS-induced shock and inhibition of circulating levels of TNFa.

Folks et al., Proc. Natl. Acad. Sci. USA, 86:2365-2368 (1989) suggests that TNF is implicated in the stimulation of viral replication of latent HIV in T-cell and macrophage lines which can be induced by TNF.

35 Osborn et al., Proc. Natl. Acad. Sci. USA 86:2336-2340 (1989) suggests that a molecular mechanism for the virus inducing activity of TNF is due to TNF's ability to activate a gene regulatory protein (NF-kB) found in the cytoplasm of cells, which promotes HIV replication through binding to a viral regulatory gene sequence (LTR)

Yale University, European Patent Application Publication Number 0,230,574 A2, published August 6, 1987, claims a method for producing pharmaceutical compositions for treating patients infected with LAV/HTLV III virus wherein such composition contains a compound which inhibits the production and/or the activity of mononuclear cell derived cytotoxic factors, such as lymphotoxin, tumor necrosis factor, leukoregulin and natural killer cytotoxic factor.

SUMMARY OF THE INVENTION

This invention relates to a method of treating a human afflicted with a human immunodeficiency virus (HIV), which comprises administering to such human an effective, monokine activity interfering amount of a monokine activity interfering agent.

DETAILED DESCRIPTION OF THE INVENTION

The human acquired immune deficiency syndrome (AIDS) results from the infection of T lymphocytes with Human Immunodeficiency Virus (HIV). HIV entry into the T lymphocyte requires T lymphocyte activation. Other viruses, such as HIV-1, HIV-2 infect T lymphocytes after T Cell activation and such virus protein expression and/or replication is mediated or maintained by such T cell activation, for example. It has now been discovered that monokines are implicated in the infection of T lymphocytes with HIV by playing a role in maintaining T lymphocyte activation. Furthermore, once an activated T lymphocyte is infected with HIV, the T lymphocyte must continue to be maintained in an activated state to permit HIV gene expression and/or HIV replication. It has now also been discovered that monokines are implicated in activated T-cell mediated HIV protein expression and/or virus replication by playing a role in maintaining T lymphocyte activation. Therefore, interference with monokine activity, such as by inhibition of monokine production, in an HIV-infected individual aids in limiting the maintenance of T cell activation thereby reducing the progression of HIV infectivity to previously uninfected cells which results in a slowing or elimination of the progression of immune dysfunction caused by HIV infection. It has now also been discovered that monokines are implicated in certain disease associated problems such as cachexia and muscle degeneration. Therefore, interference with monokine activity, such as by inhibition of monokine production, in an HIV-infected individual aids in enhancing the quality of life of HIV-infected patients by reducing the severity of monokine-mediated disease associated problems such as cachexia and muscle degeneration. Thus, it is an object of this invention to provide a method for treating an HIV-infected human by administering an effective, monokine activity interfering amount of a monokine activity interfering agent to an HIV-infected human.

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By the term "monokine" as used herein is meant any cytokine produced and secreted by a macrophage and/or monocyte of a HIV-infected human provided that such monokine is implicated in (a) the initiation and/or maintenance of T cell activation and/or activated T cell-mediated HIV gene expression and/or replication, and/or (b) any monokine-mediated disease associated problem such as cachexia or muscle degeneration. Examples of such monokines include, but are not limited to, Interleukin-1 (IL-1), Tumor Necrosis Factor-alpha (TNFa) and Tumor Necrosis Factor beta (TNFb).

As TNF-B (also known as lymphotoxin) has close structural homology with TNF-a (also known as cachectin) and since each induces similar biologic responses and binds to the same cellular receptor, both TNF-a and TNF-B are both inhibited by the compounds of the present invention and thus TNF-a and TNF-b are herein after referred to as "TNF" unless specifically delineated otherwise.

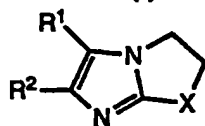
By the term "monokine activity interfering agent" as used herein is meant any compound which is useful for interfering with the activity of any monokine, such as those compounds which are useful in a method of inhibiting the production of IL-1 or TNF by monocytes and/or macrophages in a human in need thereof as claimed in any of U.S. Patent Number 4,794,114, issued December 27, 1988; U.S. Patent Number 4,788,806, issued October 18, 1988; U.S. Patent Number 4,780,470, U.S. patent application Bender *et al.*, U.S.S.N. 07/365,349, June 13, 1989, and Bender *et al.*, PCT Serial number unknown, filed contemporaneously herewith, Attorney's Docket No. 14446-1, the entire disclosures all of which are hereby incorporated by reference.

By the term "interfering with the activity of any monokine" is meant the down regulation of either the *in vivo* levels of such monokine or the activity of such monokine in a HIV-infected human to levels which interfere with T cell activation and/or activated T cell-mediated HIV gene expression and/or replication to an extent that slows disease progression.

Preferably such monokine activity interfering agent is one which is useful in inhibiting the production of any monokine, such as an agent which inhibits IL-1 production and/or TNFa production, in an HIV-infected patient. By the term "inhibiting the production of any monokine" is meant the lowering of *in vivo* levels of such monokine in a HIV-infected human to levels which interfere with T cell activation and/or activated T cell-mediated HIV gene expression and/or replication to an extent that slows disease progression. By the term "production of any monokine by monocytes and/or macrophages" is meant the *in vivo* release of such monokine by such cells.

Compounds which are useful in the method of the subject invention include those described in Bender *et al.*, U.S. Patent Number 4,794,114, issued

December 27, 1988, the entire disclosure of which is hereby incorporated by reference, which discloses a method of inhibiting the production of IL-1 by macrophages and/or monocytes in a human in need thereof by administering an effective amount of a compound of Formula (I):



wherein:

One of R¹ and R² must be 4-pyridyl and the other is selected from monohalosubstituted phenyl wherein said substituent is selected from halo or C₁₋₄ alkoxy;

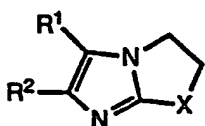
X is CH₂, CH₂CH₂ or S(O)_n; and

n is 0, 1 or 2;

or a pharmaceutically acceptable salt thereof of Compounds described in U.S.

4,794,114 which are preferred for use in the method of the subject invention include those listed in the following Table A.

TABLE A



R ¹	R ²	X	n
4-pyridyl	Fd ^a	S(O) _n	0
4-pyridyl	Fd	S(O) _n	1
4-pyridyl	Fd	S(O) _n	2
Fd	4-pyridyl	S(O) _n	0
4-pyridyl	Fd	CH ₂	-
4-pyridyl	MeOd ^b	S(O) _n	0
4-pyridyl	MeOd	S(O) _n	-
4-pyridyl	MeOd	S(O) _n	-

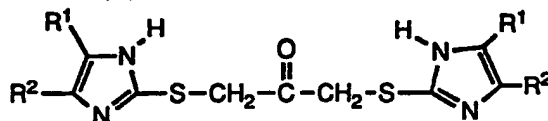
^aFd = 4-fluorophenyl

^bMeOd = 4-methoxyphenyl

The most preferred compound of TABLE A for use in the method of the subject invention is the compound known as 5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo(2,1-B)-thiazole which has been shown to inhibit both IL-1 and TNFα production by monocytes. See, Lee et al., *Int. J. Immunopharmac.*, 10(7), 835

(1988) and Badger et al., Circulatory Shock, 27(1), 51-61 (1989), the disclosures of both of which are hereby incorporated by reference.

Additional compounds which are useful in the method of the subject invention include those described in Bender et al., U.S. Patent Number 4,778,806, issued October 18, 1988, the entire disclosure of which is hereby incorporated by reference, which discloses a method of inhibiting the production of IL-1 by monocytes and/or macrophages in a human in need thereof which comprises administering to such human an effective, interleukin-1 production inhibiting amount of a compound of Formula (II):

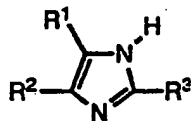


wherein:

one of R^1 and R^2 is 4-pyridyl and the other is monohalosubstituted phenyl; or a pharmaceutically acceptable salt thereof.

A compound described in U.S. 4,778,806 which is preferred for use in the method of the subject invention is one in which R^1 is 4-pyridyl and R^2 is 4-fluorophenyl.

Additional compounds which are useful in the method of the subject invention include those described in Bender et al., U.S. Patent Number 4,780,470, issued October 25, 1988, the entire disclosure of which is hereby incorporated by reference, which discloses a method of inhibiting the production of IL-1 by monocytes and/or macrophages in a human in need thereof which comprises administering to such human an effective, interleukin-1 production inhibiting amount of a compound of Formula (III):



wherein:

One of R^1 and R^2 is 4-pyridyl and the other is selected from monohalosubstituted phenyl; and

R^3 is S or $\text{SCF}_2\text{CF}_2\text{H}$;

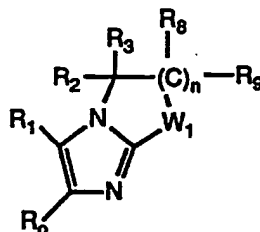
or a pharmaceutically acceptable salt thereof.

Compounds described in U.S. 4,780,470 which are preferred for use in the method of the subject invention are the ones in which R^2 is 4-pyridyl, R^1 is 4-fluorophenyl and R^3 is either SH or $\text{SCF}_2\text{SF}_2\text{H}$.

Additional compounds which are useful in the method of the subject invention include those described in U.S. patent application Bender et al., U.S.S.N.

07/365,349, June 13, 1989, and in Bender et al. PCT Application, number

unknown, Attorney's Docket Number SKB 14446-1, filed contemporaneously herewith, the entire disclosures both of which are hereby incorporated by reference, which collectively describe a method of inhibiting the production of IL-1 by monocytes and/or macrophages in a human in need thereof which comprises
 5 administering to such human an effective, interleukin-1 production inhibiting amount of a compound of Formula (IV):



wherein:

10 W₁ is -(CR₄R₅)-(CR₆R₇)-, -CR₅=CR₇-, -N=CR₇-, -S(O)_m- or -O-;
 one of R₁ and R₆ is 4-pyridyl or C₁₋₄ alkyl-4-pyridyl, provided that
 when R₁ is C₁₋₄ alkyl-4-pyridyl the alkyl substituent is located at the 2-position of
 the pyridine ring, and the other of R₁ and R₆ is

15 (a) phenyl or monosubstituted phenyl wherein said
 substituent is C₁₋₃ alkylthio, C₁₋₃ alkylsulfinyl, C₂₋₅ 1-alkenyl-1-thio,
 C₂₋₅ 1-alkenyl-1-sulfinyl, C₃₋₅ 2-alkenyl-1-thio, C₃₋₅ 2-alkenyl-1-
 sulfinyl, 1-acyloxy-1-alkylthio, C₁₋₂ alkoxy, halo, C₁₋₄ alkyl or Z
 wherein Z is -S-S-Z₁ and Z₁ is phenyl or C₁₋₉ alkyl; or

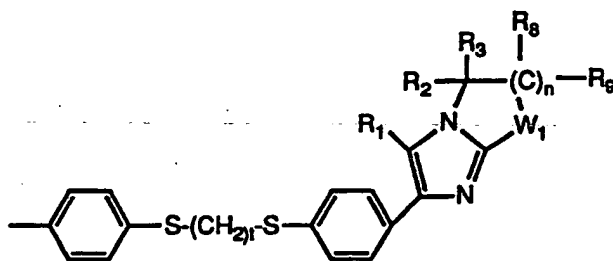
20 (b) disubstituted phenyl wherein said substituents are,
 independently, C₁₋₃ alkylthio, C₁₋₂ alkoxy, halo or C₁₋₄ alkyl; or

(c) disubstituted phenyl wherein one of said substituents is
 C₁₋₃ alkylsulfinyl, C₂₋₅ 1-alkenyl-1-thio, C₂₋₅ 1-alkenyl-1-sulfinyl,
 C₃₋₅ 2-alkenyl-1-thio, C₃₋₅ 2-alkenyl-1-sulfinyl or 1-acyloxy-1-
 alkylthio and the other is C₁₋₂ alkoxy, halo, or C₁₋₄ alkyl;

25 (d) disubstituted phenyl wherein the substituents are the same
 and are C₁₋₃ alkylsulfinyl, C₂₋₅ 1-alkenyl-1-thio, C₂₋₅ 1-alkenyl-1-
 sulfinyl, C₃₋₅ 2-alkenyl-1-thio, C₃₋₅ 2-alkenyl-1-sulfinyl or 1-
 acyloxy-1-alkylthio or wherein the substituents together form a
 methylene dioxy group; or

30 (e) monosubstituted phenyl wherein the substituent is

-9-



t is 0 or 1; W₁, R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈ and R₉ are as defined above;

provided that:

- 5 (1.) when W₁ is -(CR₄R₅)-(CR₆R₇)- then
 - n is 0 or 1; and
 - R₂, R₃, R₄, R₅, R₆, R₇, R₈, and R₉ are, independently, -H or C₁₋₂ alkyl; and
 - when R₁ or R₀ is 4-pyridyl, the other of R₁ and R₀ is other than
 - 10 mono-C₁₋₂ alkoxy-substituted phenyl or mono-halo-substituted phenyl; or
 - when n is 0, R₄ and R₅ together form an oxo; R₄ and R₅ are both fluoro, or one of R₄ and R₅ is H and the other OH; or
 - (2.) when W₁ is -CR₅=CR₇- or -N=CR₇- then
 - 15 n is 1;
 - R₃, R₅, R₇ and R₉ are, independently, -H or C₁₋₂ alkyl; and
 - R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic pyridine or pyrimidine ring;
 - 20 (3.) when W₁ is S(O)_m then
 - m is 0, 1 or 2;
 - n is 1 or 2; and
 - R₃ and R₉ are, independently, -H or C₁₋₂ alkyl;
 - R₂ and R₈ are, independently, -H or C₁₋₂ alkyl or R₂ and R₈
 - 25 together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;
- further provided that:
- (a) when R₂ and R₈ are, independently, -H or C₁₋₂ alkyl and R₁ or R₀ is 4-pyridyl, then the other of R₁ and R₀ is other
 - 30 than mono-C₁₋₂ alkoxy-substituted phenyl or mono-halo-substituted phenyl; and
 - (b) when R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring, then m is 0 and n is 1; and

-10-

(4) when W_1 is -O- then

n is 1;

R_3 and R_9 are, independently, -H or C_{1-2} alkyl; and

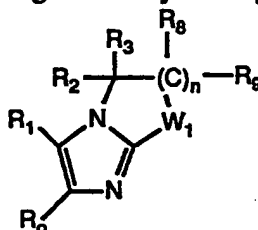
R_2 and R_8 together represent a double bond in the B ring such that the

5

B ring is an aromatic oxazole ring; or

the pharmaceutically acceptable salts thereof.

Compounds of the general bicyclic ring structure



10 are herein defined to have two rings, the first ring, containing the dihydroimidazole and substituent groups R_0 and R_1 , is defined as the "A" ring. The saturated or unsaturated ring containing the W_1 term and $(C)_n$ term, etc. is defined as the "B" ring.

15 Compounds described in Bender *et al.*, U.S.S.N. 07/365,349 June 13, 1989, and in Bender *et al.* PCT Serial number unknown, filed contemporaneously herewith, Attorneys Docket Number SKB 14446-1, which are preferred for use in the method of the subject invention include those wherein:

W_1 is $-(CR_4R_5)-(CR_6R_7)-$, $-CR_5=CR_7-$, or $-S(O)_m-$;

20 one of R_1 and R_0 is 4-pyridyl or C_{1-2} alkyl-4-pyridyl, provided that when R_1 is C_{1-2} alkyl-4-pyridyl the alkyl substituent is located at the 2-position of the pyridine ring, and the other of R_1 and R_0 is

25 (a) monosubstituted phenyl wherein said substituent is C_{1-2} alkylthio, C_{1-2} alkylsulfinyl, 1-acyloxy-1-alkylthio, C_{1-2} alkoxy or halo, or

(b) disubstituted phenyl wherein said substituents are, independently, C_{1-2} alkylthio or C_{1-2} alkoxy, or

30 (c) disubstituted phenyl wherein one of said substituents is C_{1-2} alkylsulfinyl or 1-acyloxy-1-alkylthio and the other is C_{1-2} alkoxy, or

(d) disubstituted phenyl wherein the substituents are the same and are C_{1-2} alkylsulfinyl or 1-acyloxy-1-alkylthio or wherein the substituents together form a methylene dioxy group;

provided that:

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- (1.) when W_1 is $-(CR_4R_5)-(CR_6R_7)-$ then
 n is 0 or 1; and
 $R_2, R_3, R_4, R_5, R_6, R_7, R_8,$ and R_9 are -H; and
 when R_1 or R_0 is 4-pyridyl, the other of R_1 and R_0 is other than
 mono- C_{1-2} alkoxy-substituted phenyl or mono-flouro-substituted
 phenyl;
- (2.) when W_1 is $-CR_5=CR_7-$ then
 n is 1;
 R_3, R_5, R_7 and R_9 are -H; and
 R_2 and R_8 together represent a double bond in the B ring such that the
 B ring is an aromatic pyridine ring;
- (3.) when W_1 is $S(O)_m$ then
 m is 0, 1 or 2;
 n is 1 or 2; and
 R_3 and R_9 are -H;
 R_2 and R_8 are -H or R_2 and R_8 together represent a double bond in
 the B ring such that the B ring is an aromatic thiazole ring;
 further provided that:
 (a) when R_2 and R_8 are -H and R_1 or R_0 is 4-pyridyl, then
 the other of R_1 and R_0 is other than mono- C_{1-2} alkoxy-
 substituted phenyl or mono-halo-substituted phenyl; and
 (b) when R_2 and R_8 together represent a double bond in the B
 ring such that the B ring is an aromatic thiazole ring, then m is
 0 and n is 1; and
- (4.) when W_1 is -O- then
 n is 1;
 R_3 and R_9 are -H; and
 R_2 and R_8 together represent a double bond in the B ring such that the
 B ring is an aromatic oxazole ring;
- or a pharmaceutically acceptable salt thereof.

Compounds described in Bender *et al.*, U.S.S.N. 07/365,349, filed
 June 13, 1989, and in Bender *et al.*, PCT application, number unknown, Attorney's
 Docket Number SKB 14446-1, filed contemporaneously herewith, which are
 especially preferred for use in the method of the subject invention include the
 following.

2-(4-Methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-
 [5H]-pyrrolo-[1,2-a]-imidazole;

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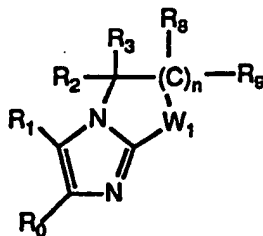
- 2-(4-Methylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 2-(4-Ethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 5 2-(4-Ethylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 2-(4-Methylthiophenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 10 2-(4-Methylsulfinylphenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 2-(4-Methoxyphenyl)-3-(4-pyridyl)-imidazo-[1,2-a]-pyridine;
- 5-(3,4-(M ethylenedioxy)phenyl)-6-(4-pyridyl)-2,3-dihydroimidazo-[2,1-b]-thiazole;
- 15 2-(4-Methoxyphenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 2-(4-Acetoxymethylthiophenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 20 6-(4-Methylthiophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole;
- 5-(4-Methylthiophenyl)-6-(4-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole;
- 3-(4-Methylthiophenyl)-2-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]imidazole;
- 25 2-(4-Propylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 2-(4-Methylthiophenyl)-3-(4-(2-ethyl)pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole; and
- 2-(4-Mercaptophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole disulfide.
- 30

Compounds which are useful in the method of the subject invention for inhibition of TNF include those described in Bender *et al.* PCT Application, number unknown, Attorneys Docket Number SKB 14446-1, filed

35 contemporaneously herewith, the entire disclosure of which is hereby incorporated by reference, which describes a method of inhibiting the production of TNF by monocytes and/or macrophages in a human in need thereof which comprises

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administering to such human an effective, TNF production inhibiting amount of a compound of Formula (V):



5 wherein:

W_1 is $-(CR_4R_5)-(CR_6R_7)-$, $-CR_5=CR_7-$, $-N=CR_7-$, $-S(O)_m-$ or $-O-$;

one of R_1 and R_0 is 4-pyridyl or C_{1-4} alkyl-4-pyridyl,

provided that when R_1 is C_{1-4} alkyl-4-pyridyl the alkyl substituent is located at the 2-position of the pyridine ring, and the other of R_1 and R_0 is

10

(a) phenyl or monosubstituted phenyl wherein said substituent is C_{1-3} alkylthio, C_{1-3} alkylsulfinyl, C_{2-5} 1-alkenyl-1-thio, C_{2-5} 1-alkenyl-1-sulfinyl, C_{3-5} 2-alkenyl-1-thio, C_{3-5} 2-alkenyl-1-sulfinyl, 1-acyloxy-1-alkylthio, C_{1-2} alkoxy, halo, C_{1-4} alkyl or Z wherein Z is $-S-S-Z_1$ and Z_1 is phenyl or C_{1-9} alkyl; or

15

(b) disubstituted phenyl wherein said substituents are, independently, C_{1-3} alkylthio, C_{1-2} alkoxy, halo or C_{1-4} alkyl; or

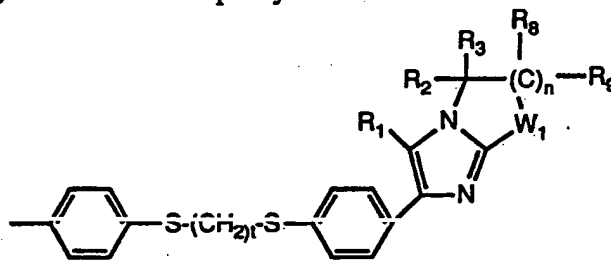
(c) disubstituted phenyl wherein one of said substituents is C_{1-3} alkylsulfinyl, C_{2-5} 1-alkenyl-1-thio, C_{2-5} 1-alkenyl-1-sulfinyl, C_{3-5} 2-alkenyl-1-thio, C_{3-5} 2-alkenyl-1-sulfinyl or 1-acyloxy-1-alkylthio and the other is C_{1-2} alkoxy, halo, or C_{1-4} alkyl;

20

(d) disubstituted phenyl wherein the substituents are the same and are C_{1-3} alkylsulfinyl, C_{2-5} 1-alkenyl-1-thio, C_{2-5} 1-alkenyl-1-sulfinyl, C_{3-5} 2-alkenyl-1-thio, C_{3-5} 2-alkenyl-1-sulfinyl or 1-acyloxy-1-alkylthio or wherein the substituents together form a methylene dioxy group; or

25

(e) monosubstituted phenyl wherein the substituent is



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t is 0 or 1; W, R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈ and R₉ are as defined above;

provided that:

(1.) when W₁ is -(CR₄R₅)-(CR₆R₇)- then

5 n is 0; R₄ and R₅ together may form an oxo; R₄ and R₅ are both fluoro, or one of R₄ and R₅ is H and the other OH; or

(2.) when W₁ is -CR₅=CR₇- or -N=CR₇- then

n is 1;

10 R₃, R₅, R₇ and R₉ are, independently, -H or C₁₋₂ alkyl; and

R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic pyridine or pyrimidine ring;

(3.) when W₁ is S(O)_m then

m is 0, 1 or 2;

15 n is 1 or 2; and

R₃ and R₉ are, independently, -H or C₁₋₂ alkyl;

R₂ and R₈ are, independently, -H or C₁₋₂ alkyl or R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;

20 further provided that:

(a) when R₂ and R₈ are, independently, -H or C₁₋₂ alkyl and R₁ or R₀ is 4-pyridyl, then the other of R₁ and R₀ mono-fluoro-substituted phenyl; and

25 (b) when R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring, then m is 0 and n is 1; and

(4) when W₁ is -O- then

n is 1;

30 R₃ and R₉ are, independently, -H or C₁₋₂ alkyl; and

R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic oxazole ring;

or a pharmaceutically acceptable salt thereof.

Additional compounds which are preferred for use in the method of the subject invention include those wherein:

35 W₁ is -(CR₄R₅)-(CR₆R₇)-, -CR₅=CR₇-, or -S(O)_m-;

one of R₁ and R₀ is 4-pyridyl or C₁₋₂ alkyl-4-pyridyl, provided that when R₁ is C₁₋₂ alkyl-4-pyridyl the alkyl substituent is located at the 2-position of the pyridine ring, and the other of R₁ and R₀ is

-15-

(a) monosubstituted phenyl wherein said substituent is C₁₋₂ alkylthio, C₁₋₂ alkylsulfinyl, 1-acyloxy-1-alkylthio, C₁₋₂ alkoxy or halo, or

(b) disubstituted phenyl wherein said substituents are, independently, C₁₋₂ alkylthio or C₁₋₂ alkoxy, or

(c) disubstituted phenyl wherein one of said substituents is C₁₋₂ alkylsulfinyl or 1-acyloxy-1-alkylthio and the other is C₁₋₂ alkoxy, or

(d) disubstituted phenyl wherein the substituents are the same and are C₁₋₂ alkylsulfinyl or 1-acyloxy-1-alkylthio or wherein the substituents together form a methylene dioxy group;

provided that:

(1.) when W₁ is -CR₅=CR₇- then

n is 1;

R₃, R₅, R₇ and R₉ are -H; and

R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic pyridine ring;

(2.) when W₁ is S(O)_m then

m is 0, 1 or 2;

n is 1 or 2; and

R₃ and R₉ are -H;

R₂ and R₈ are -H or R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;

further provided that:

(a) when R₂ and R₈ are -H and R₁ or R₀ is 4-pyridyl, then the other of R₁ and R₀ is other than mono-flouro-substituted phenyl; and

(b) when R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring, then m is 0 and n is 1; and

(4) when W₁ is -O- then

n is 1;

R₃ and R₉ are -H; and

R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic oxazole ring.

Additional compounds which are useful in the method of the subject invention because they are inhibitors of TNF and IL-1 include 2-(4-Methoxyphenyl)-3-

(4-pyridyl)-7-oxo-5,6-dihydro-[7H]-pyrrolo-[1,2-a]-imidazole; 5,6-dihydro-2-(4-Methoxyphenyl)-3-(4-pyridyl)-[7H]-pyrrolo-[1,2-a]-imidazole-7-ol; and 5,6-dihydro-7,7-difluoro-2-(4-Methoxyphenyl)-3-(4-pyridyl)-[7H]-pyrrolo-[1,2-a]-imidazole are described in Tetrahedron Letter, Vol. 30, No. 48, pp. 6599-6602 (1989) the entire disclosure of which is hereby incorporated by reference.

Especially preferred compounds for use in the method of the subject invention are

- 2-Phenyl-3-pyridyl-6,7-dihydro-[5H]-pyrrolo-[1,2-a]imidazole;
- 2-4-Bromophenyl-3-pyridyl-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
- 10 2-(4-Pyridyl)-3-(4-fluorophenyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a] imidazole;
- 2-(4-Fluorophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;
- 2-(4-Methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;
- 15 2-(4-Pyridyl)-3-(4-methylthiophenyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;
- 2-(4-Methylthiophenyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;
- 2-(4-Methoxyphenyl)-3-(4-pyridyl)-7-oxo-5,6-dihydro-7H-pyrrolo-[1,2-a]imidazole;
- 20 [1,2-a]imidazole;
- 5,6-Dihydro-2-(4-methoxyphenyl)-3-(4-pyridinyl)-[7H]-pyrrolo[1,2-a]-imidazole-7-ol;
- 2-(4-Acetoxymethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro[5H]pyrrolo-[1,2-a]-imidazole;
- 25 2-(4-Methoxyphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]imidazole;
- 6-(4-Fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole;
- 6-(4-Fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo-[2,1-b]thiazole-1-oxide;
- 30 6-(4-Fluorophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole-1,1-dioxide;
- 5-(4-Fluorophenyl)-6-(4'-pyridyl)-2,3-dihydroimidazo-[2,1-b]thiazole;
- 2-(4-Methylsulfoxyphenyl)-3-(4-pyridyl)-6,7-dihydro[5H]-pyrrolo-[1,2-a]imidazole;
- 35 2-(4-Ethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole; or

2-(4-Ethylsulfonylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole.

5 This invention relates to a method of treating a human afflicted with a human immunodeficiency virus, which comprises administering to such human an effective, monokine activity interfering amount of a monokine activity interfering agent. The monokine activity interfering agent is administered to a HIV-infected human in an amount sufficient to interfere with the production or activity of any monokine (a) implicated in T lymphocyte activation to levels which interfere with T
10 cell activation and/or activated T cell-mediated HIV gene expression and/or replication to an extent that slows disease progression and/or (b) implicated in monokine-mediated disease associated problems such as cachexia and muscle degeneration to levels which improve the quality of the life of the HIV-infected individual. Preferably such monokine activity interfering agent is one which inhibits IL-1 production, TNFa
15 or TNFb production by monocytes and/or macrophages in an HIV-infected patient. In an HIV-infected human manifesting immune dysfunction, treatment with an effective amount of a monokine activity interfering agent will initially result in a slowing of the rate of T cell depletion, thereby slowing disease progression. It is expected that T cell depletion will gradually cease and that T cell counts and T4/T8
20 ratios will begin to normalize. In an HIV-infected human not yet manifesting immune dysfunction, treatment with an effective amount of a monokine activity interfering agent will result in a slowing of the rate of T cell depletion, thereby slowing disease progression and delaying immune dysfunction manifestation. In an HIV-infected human manifesting monokine-mediated disease associated problems such as cachexia
25 or muscle degeneration, treatment with an effective amount of a monokine activity interfering agent will initially result in a slowing of the rate of the progression of the disease associated problem, thereby slowing disease progression. It is expected that the progression of the disease associated problem will eventually cease and reverse, thereby enhancing the quality of life of the HIV-infected individual treated in such a
30 manner.

It will be recognized by one of skill in the art that the actual amount of a monokine activity interfering agent required for therapeutic effect will, of course, vary with the agent chosen, the route of administration desired, the nature and severity of the HIV-infection and the particular condition of the HIV-infected human
35 undergoing treatment, and is ultimately at the discretion of the physician. It will also be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a monokine activity interfering agent will be determined by the nature and extent of the condition being treated, the form, route and site of

administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of a monokine activity interfering agent given per day for a defined number of days, can be
5 ascertained by those skilled in the art using conventional course of treatment determination tests.

The monokine activity interfering agent is administered orally, topically, parenterally or by inhalation in conventional dosage forms prepared by combining such agent with standard pharmaceutical carriers according to conventional
10 procedures in an amount sufficient to produce therapeutic monokine activity interfering activity.

The pharmaceutical carrier employed can be readily determined by one of skill in the art who will recognize that such determination will depend upon various well-known factors such as the nature, quantity and character of the particular
15 monokine activity interfering agent being employed and the form and route of administration desired.

The method of the subject invention may be carried out by delivering the monokine activity interfering agent parenterally, orally, topically or by inhalation depending upon various factors such as the nature of the agent and the desired site of
20 inhibition.

In general, an initial treatment regimen can be copied from that known to be effective in interfering with monokine activity for the particular monokine activity interfering agent employed. Treated individuals will be regularly checked for T cell numbers and T4/T8 ratios and/or for progression of monokine-mediated
25 disease associated problems such as cachexia or muscle degeneration. If no effect is seen following the normal treatment regimen, then the amount of the monokine activity interfering agent administered is increased, e.g., by fifty percent per week.

As stated above, the method of the subject invention may be carried out by delivering the monokine activity interfering agent topically. By topical
30 administration is meant non-systemic administration and includes the application of a monokine activity interfering agent externally to the epidermis, to the buccal cavity and instillation of such a compound into the ear, eye and nose, and where the compound does not significantly enter the blood stream.

A suitable monokine activity interfering dose of any IL-1 production
35 inhibiting compound disclosed in any of the method of use claims of U.S. Patent Numbers 4,794,114, 4,778,806 and 4,780,470. U.S. patent application Bender et al., U.S.S.N. 07/365,349, June 13, 1989, and Bender et al., PCT Application, number unknown, Attorney's Docket Number SKB 14446-1, filed

contemporaneously herewith, is 15 mg to 500 mg of base for topical administration, the most preferred dosage being 1 mg to 100 mg, for example, 5 to 25 mg administered two or three times daily. The daily topical dosage regimen will preferably be from about 2 mg to about 10 mg per site of administration.

5 A suitable monokine activity interfering dose of any TNF production inhibiting compound is from about 1 mg to about 1000 mg of base for topical administration, the most preferred daily dosage being 15 mg to 500 mg, the single dosage range being about 5mg to 160 mg. The daily topical dosage regimen will preferably be from about 2 mg to about 10 mg per site of administration.

10 As stated above, the method of the subject invention may be carried out by delivering the monokine activity interfering agent by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. A suitable monokine
15 activity interfering dose herewith, administered by inhalation is from about 1 mg to about 100 mg per day. More detailed information about inhalation formulation of such compounds is outlined in the specifications for any of the IL-1 production inhibiting compounds disclosed in the method of use claims of U.S. Patent Numbers 4,794,114, 4,778,806 and 4,780,470, U.S. patent application Bender *et al.*,
20 U.S.S.N. 07/365,349, filed June 13, 1989, and Bender *et al.*, PCT Application, number unknown, Attorney's Docket Number SKB 14446-1, filed contemporaneously herewith..

 A suitable monokine activity interfering dose of any TNF production inhibiting compound administered by inhalation is from about 1 mg to about 1000 mg
25 per day. More preferably from about 10mg to about 100mg per day.

 As stated above, the method of the subject invention may be carried out by delivering the monokine activity interfering agent parenterally. The term 'parenteral' as used herein includes intravenous, intramuscular, subcutaneous intranasal, intrarectal, intravaginal or intraperitoneal administration. The
30 subcutaneous and intramuscular forms of parenteral administration are generally preferred. Appropriate dosage forms for such administration may be prepared by conventional techniques. A suitable monokine activity interfering dose, and more detailed information about parenteral formulation of such compounds is outlined in the specifications of any IL-1 production inhibiting compound disclosed any of the
35 method of use claims of in U.S. Patent Numbers 4,794,114, 4,778,806 and 4,780,470, U.S. patent application Bender *et al.*, U.S.S.N. 07/365,349, filed June 13, 1989, and in Bender *et al.*, PCT serial number unknown, Attorney's Docket Number SKB 14446-1, filed contemporaneously herewith. The dose administered

parenterally will preferably be from about 1 to about 100 mg per kilogram (kg) of total body weight, most preferably from about 3 to about 60 mg/kg per day.

5 A suitable monokine activity interfering dose of any TNF production inhibiting compound administered parenterally will preferably be from about 1 to about 100 mg per kilogram (kg) of total body weight, most preferably from about 5 to about 80 mg/kg per day.

10 As stated above, the method of the subject invention may be carried out by delivering the monokine activity interfering agent orally. Appropriate dosage forms for such administration may be prepared by conventional techniques. A suitable monokine activity interfering dose of any IL-1 production inhibiting compound disclosed in any of the method of use claims of U.S. Patent Numbers 4,794,114, 4,778,806 and 4,780,470, U.S. patent application Bender *et al.*, U.S.S.N. 07/365,349, June 13, 1989, and in Bender *et al.*, PCT Application Number unknown, Attorney's Docket Number SKB 14446-1, filed
15 contemporaneously herewith, administered orally will preferably be from about 5 to about 100 mg/kilogram of total body weight per day.

20 As stated above, the method of the subject invention may be carried out by delivering the monokine activity interfering agent orally. Appropriate dosage forms for such administration may be prepared by conventional techniques. A suitable monokine activity interfering dose of any TNF production inhibiting compound will preferably be from about 1 to about 100 mg per kilogram (kg) of total body weight, most preferably from about 5 to about 80 mg/kg per day. More detailed information about topical, oral, inhalation and parenteral dosage formulations for the TNF inhibiting compounds of the subject application is outlined in the specifications
25 of U.S. patent application Bender *et al.*, U.S.S.N. 07/365,349, filed June 13, 1989 and Bender *et al.*, PCT Application number unknown, Attorney's Docker Number SKB SKB 14446-1, filed contemporaneously herewith.

30 This invention also relates to a method of treating HIV infection in a human infected with HIV which comprises administering to such human an effective amount of 5-(4-pyridyl)-6-(4-fluorophenyl)-2,3-dihydroimidazo-(2,1-b)-thiazole. By the term "5-(4-pyridyl)-6-(4-fluorophenyl)-2,3-dihydroimidazo-(2,1-b)-thiazole" as used herein is meant both the base form of the compound as well as all pharmaceutically acceptable salts thereof.

35 This invention also relates to a method of treating HIV infection in a human infected with HIV which comprises administering to such human an effective amount of 2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole as well as all pharmaceutically acceptable salts thereof.

A method for the preparation of 5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo-(2,1-b)-thiazole (including all pharmaceutically acceptable salt forms thereof) is described by U.S. Patent 4,175,127, issued November 20, 1979, and an improved method for its preparation is described by U.S. Patent 4,803,279, issued February 9, 1989, the entire disclosures of both of which patents are hereby incorporated by reference. A method for the preparation of 2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole, 4-alkylthiophenyl and 4-alkylsulfanylphenyl derivatives is specifically described in US Patent No. 4,719,218 issued January 12, 1988 and is hereby incorporated by reference.

The compound 5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo-(2,1-B)-thiazole (hereinafter referred to as "Compound 1") and 2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole, (hereinafter referred to as "Compound 2") are administered to a HIV-infected human in an amount sufficient to have a therapeutic effect on such individual's infection by enhancing the quality of life of the infected individual by slowing the progression of the disease and/or by ameliorating HIV associated conditions such as cachexia and muscle degeneration. It will be recognized by one of skill in the art that the actual amount of Compounds 1 or 2 as well as other pyrrolo-[2,1-a]-imidazoles and imidazo-[2,1-b]-thiazoles of the compounds described herein required for therapeutic effect will, of course, vary with the route of administration desired, the nature and severity of the HIV-infection and the particular condition of the HIV-infected human undergoing treatment, and is ultimately at the discretion of the physician. It will also be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of Compounds 1 or 2 will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of Compounds 1 or 2 given per day for a defined number of days, can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Compounds 1 or 2 are administered orally, topically, parenterally or by inhalation in conventional dosage forms prepared by combining such agent with standard pharmaceutical carriers according to conventional procedures.

The pharmaceutical carrier employed can be readily determined by one of skill in the art who will recognize that such determination will depend upon various well-known factors such as the form and route of administration desired.

As stated above, the method of the subject invention may be carried out by delivering Compounds 1 or 2 topically. By topical administration is meant

non-systemic administration and includes the application of Compounds 1 or 2 externally to the epidermis, to the buccal cavity and instillation of such a compound into the ear, eye and nose, and where the compound does not significantly enter the blood stream. A suitable dose of Compounds 1 or 2 is 1.5 mg to 100 mg of base for
5 topical administration, the most preferred dosage being 1 mg to 500 mg, for example 5 to 25 mg administered two or three times daily. The daily topical dosage regimen will preferably be from about 2 mg to about 10 mg per site of administration. More detailed information about topical formulation of Compound 1 is outlined in the specification of U.S. Patent Number 4,794,114.

1 0 As stated above, the method of the subject invention may be carried out by delivering Compounds 1 or 2 by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. A suitable dose of Compounds 1 or 2
1 5 administered by inhalation is from about 1 mg/kg to about 100 mg/kg per day. More detailed information about inhalation formulation of Compound 1 is outlined in the specification of U.S. Patent Numbers 4,794,114.

As stated above, the method of the subject invention may also be carried out by delivering Compounds 1 or 2 parenterally. The term 'parenteral' as
2 0 used herein includes intravenous, intramuscular, subcutaneous intranasal, intrarectal, intravaginal or intraperitoneal administration. The subcutaneous and intramuscular forms of parenteral administration are generally preferred. Appropriate dosage forms for such administration may be prepared by conventional techniques. A suitable dose of Compounds 1 or 2 administered parenterally will preferably be from about 1 to
2 5 about 100 mg per kilogram (kg) of total body weight, most preferably from about 2 to about 60 mg/kg per day. More detailed information about parenteral formulation of Compound 1 is outlined in the specification of U.S. Patent Number 4,794,114.

As stated above, the method of the subject invention may also be carried out by delivering Compounds 1 or 2 orally. Appropriate dosage forms for
3 0 such administration may be prepared by conventional techniques. A suitable dose of Compounds 1 or 2 administered orally will preferably be from about 1 to about 100 mg/kilogram of total body weight per day. More preferably from about 2mg/kg to about 60mg/kg per day. More detailed information about oral formulation of Compound 1 is outlined in the specification of U.S. Patent Numbers 4,794,114.

3 5 This invention relates to the use of a compound of Formula (I), Formula (II), Formula (III), Formula (IV), or Formula (V) or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment of a human afflicted with a human immunodeficiency virus (HIV), which comprises

administering to such human an effective amount of a monokine activity interfering agent of Formula's (I) -(V).

UTILITY EXAMPLES

EXAMPLE A

5 Two models of endotoxin shock have been utilized to determine in vivo TNF activity and are described below. The actual protocol used in the models is described in Utility Model Examples A and B set out below, i.e. P. Acnes model and LPS-GAL model. In these models protection from the lethal effects of endotoxin shock is provided by the compound 6-(4'-Fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo-
10 [2,1-b]-thiazole, (herein called Compound 1) and 2-(4-methyl-sulfoxylphenyl)-3-(4-pyridyl)-6,7-dihydro[5H]-pyrrolo-[1,2-a]-imidazole (herein called Compound 2). The data in Figures 1-5 clearly demonstrates the ability of Compounds 1 and 2 to reduce the in vivo level of tumor necrosis factor (TNF).

Compound 2 shows a reduction in serum TNF levels in the P acnes/LPS treated
15 mice model as depicted by the data shown in Figure 1, which demonstrates decreased levels of in vivo TNF relative to increased oral dosage of Compound 2. Figure 2 demonstrates inhibition of TNF production, also in the P. acnes/LPS Model for both Compounds 1 and 2.

Figure 3 demonstrates that (intra-peritoneal) i.p.injection of Compound 1 on
20 inhibits TNF production in the LPS-Gal mouse model of endotoxic shock. Figure 4 shows a comparison of the reduction of serum TNF levels at 100 mg/kg for both Compounds 1 and 2 in the LPS-GAL Model. Figure 5 demonstrates 100% survival rate of the animals with endotoxic shock in the LPS-GAL model after treatment with Compounds 1 and 2 compared to only a 30% survival rate of the animals in the control
25 group.

It has also been determined, using one or both of the in vivo assays described herein, that 2-(4-Ethylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole, 2-(4-methylthiolphenyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole, and 6-(4-Fluorophenyl)-5-(4-pyridyl)-2,3-
30 dihydroimidazo[2,1-b]thiazole-1,1-dioxide also inhibited in vivo TNF levels as well as protected the animals from endotoxic induced shock.

The data shown herein demonstrate that the compounds of the present invention inhibit TNF production in a mammal. Therefore, the compounds of the present invention are useful as monokine activity interfering agents, e.g. they are useful
35 in inhibiting the production of tumor necrosis factor (TNF) by monocytes or macrophages in a human.

UTILITY EXAMPLE MODEL A

Endotoxin Shock in D-gal-Sensitized Mice

The protocol used to test the compound of the method of the subject invention was essentially as has been described in Galanos et al., Proc. Nat'l Acad. Sci USA, 76:5939-43 (1979) whose disclosure is herein incorporated by reference. Briefly, D-gal (D(+) Galactosidase) sensitizes various strains of mice to the lethal effects of endotoxin. The administration of D-gal (300-500mg/kg) intra-venously (i.v.) sensitizes the mice to doses of Lipopolysaccharide(LPS) as low as 0.1mg. Briefly, male C57BL/6 mice, obtained from Charles River Laboratories (Stone Ridge, New York, USA) of 6-12 weeks of age were injected i.v. with 0.1 mg of LPS from Salmonella typhosa (Difco Laboratories, Detroit, Michigan, USA) admixed with D(+)-gal (Sigma; 500 mg/kg) in 0.20-0.25 ml pyrogen-free saline. Compounds to be tested were administered at various times prior to or following the i.v. injection of LPS/D-gal. In this model, the control animals usually die 5-6 hr. following the injection of LPS, although on occasion deaths are seen between 24 and 48 hr.

UTILITY MODEL EXAMPLE B

1 5 Endotoxin Shock in P. acnes-Sensitized Mice

This model is a modification of the previously described protocol for the in vivo induction of TNF as described in Haranaka et al., Cancer Immunol Immunother, 18:87-90, (1984). Treatment with Propionibacterium Acnes (P. acnes) (1mg/animal i.p.) renders mice susceptible to the lethal effects of LPS injected 10 days later.

2 0 P. acnes was purchased from Burroughs Wellcome (Triangle Park, North Carolina, USA), and 1 microgram (ug) of the heat-killed bacteria was administered in 0.5 ml pyrogen-free saline by intraperitoneal (i.p.) injection to male C57BL/6 mice, obtained from Charles River Laboratories (Stone Ridge, New York, USA) of 6-12 weeks of age. Ten days later, the mice were injected i.v. with 1 mg LPS in 0.25 ml saline. Compounds to be tested were administered at various times prior to or following the injection of LPS. The survival of animals was monitored for 1 week.

2 5 Measurement of TNF Activity

Plasma levels of TNF were measured using a modification of the basic sandwich ELISA method described in Winston et al., Current Protocols in Molecular Biology, Pg. 11.2.1, Ausubel et al., Ed. (1987) John Wiley and Sons, New York, USA. The Elisa employed a hamster monoclonal anti-mouse TNF (Genzyme, Boston, MA, USA) as the capture antibody and a polyclonal rabbit anti-murine TNF (Genzyme, Boston, MA, USA) as the detecting antibody. TNF levels in rat samples were calculated from a standard curve generated with recombinant murine TNF (Genzyme, Boston, MA, USA). TNF levels determined by ELISA correlated with levels detected by the L929 bioassay of Ruff et. al., J. Immunol, 125:1671-1677 (1980), with 1 Unit of activity in the bioassay corresponding to 70 picograms (pg) of TNF in the ELISA. The ELISA detected levels of TNF down to 25 pg/ml.

UTILITY EXAMPLES

In the following table of the Utility Examples, the following abbreviations are employed:

	<u>ABBREVIATION</u>	<u>FORMULA (I) COMPOUNDS</u>
5	Compound 1	6-(4-Fluorophenyl)-5-(4'-pyridyl)- 2,3-dihydroimidazo[2,1-b]-thiazole;
	Compound 2	2-(4-Methylsulfoxyphenyl)-3-(4- pyridyl)-6,7-dihydro[5H]-pyrrolo- [1,2-a]imidazole;
10	Compound 3	2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7- dihydro-[5H]-pyrrolo[1,2-a]-imidazole;
	Compound 4	2-phenyl-3-pyridyl-6,7-dihydro- [5H]-pyrrolo[1,2-a]imidazole;
15	Compound 5	2-p-bromophenyl-3-pyridyl-6,7- dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;
	Compound 6	3-(4-fluorophenyl)-2-(4-pyridyl)-6,7- dihydro-[5H]-pyrrolo-[1,2-a]imidazole;
	Compound 7	2-(4-Methoxyphenyl)-3-(4-pyridyl)- 7-oxo-5,6-dihydro-7-H-pyrrolo- [1,2-a]imidazole;
20	Compound 8	5,6-dihydro-2-(4-methoxyphenyl)- 3-(4-pyridinyl)-[7H]-pyrrolo[1,2-a]- imidazole-7-ol;
25	Compound 9	2-(4-Acetoxymethylthiophenyl)-3- (4-pyridyl)-6,7-dihydro[5H]pyrrolo- [1,2-a]-imidazole;
	Compound 10	2-(4-Methoxyphenyl)-3-(4-pyridyl)- 6,7-dihydro-[5H]-pyrrolo- [1,2-a]imidazole;
30	Compound 11	2-(4-Fluorophenyl)-3-(4-pyridyl)- 6,7-dihydro-[5H]-pyrrolo[1,2-a]- imidazole;
	Compound 12	6-(4-Fluorophenyl)-5-(4'-pyridyl)- 2,3-dihydroimidazo[2,1-b]thiazole-1- oxide;
35	Compound 13	5-(4-Fluorophenyl)-6-(4'-pyridyl)- 2,3-dihydroimidazo[2,1-b]thiazole;

- Compound 14 6-(4-Fluorophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole-1,1-dioxide;
- Compound 15 2-(4-Ethylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro[5H]pyrrolo[1,2-a]imidazole;
- 5 Compound 16 2-(4-Methylthiophenyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro[5H]pyrrolo[1,2-a]imidazole.

EXAMPLE A

Inhibitory Effect of Compounds on *in vitro* TNF Production by Human Monocytes

10

The effects of the compounds enumerated above on the *in vitro* production of TNF by human monocytes was examined using the following protocol.

- Bacterial lipopolysaccharide (LPS) was used to induce TNF production by human peripheral blood monocytes. TNF activity was measured by a modification of
- 15 Winston et al., ELISA, described below. Human peripheral blood monocytes were isolated and purified from either fresh blood preparations from volunteer donors, from blood bank buffy coats, or from plateletpheresis residues according to the procedure of Colotta et al., *J. Immunol.*, 132, 936 (1984). 1×10^6 of such monocytes were plated in 24-well plates at a concentration of 1-2 million/ml per well. The cells were allowed
- 20 to adhere for 2 hours, after which time non-adherent cells were removed by gentle washing. Test compounds were then added to the cells for 1 hour (hr) before the addition of LPS (50 ng/ml), and the cultures were incubated at 37°C for an additional 24 hours. At the end of the incubation period, culture supernatants were removed and clarified of cells and all debris. Culture supernatants were immediately assayed for
- 25 TNF levels in the manner described below.

MEASUREMENT OF HUMAN TNF:

- Levels of TNF were measured using a modification of the basic sandwich ELISA assay method described in Winston et al., Current Protocols in Molecular Biology, Page 11.2.1, Ausubel et al., Ed. (1987) John Wiley and Sons, New York,
- 30 USA. The Elisa employs a murine monoclonal anti-human TNF antibody, described below, as the capture antibody and a polyclonal rabbit anti-human TNF, described below, as the second antibody. For detection, a peroxidase-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, Indiana, USA, Catalog # 605222) was added followed by a substrate for peroxidase (1mg/ml orthophenylenediamine with
- 35 0.1% urea peroxide). TNF levels in samples were calculated from a standard curve generated with recombinant human TNF produced in E. Coli (obtained from SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA).

PRODUCTION OF ANTI-HUMAN TNF ANTIBODIES:

Monoclonal antibodies to human TNF were prepared from spleens of BALB/c mice immunized with recombinant human TNF using a modification of the method of Kohler and Millstein, Nature 256:495 (1975), the entire disclosure of which is hereby incorporated by reference. Polyclonal rabbit anti-human TNF antibodies were prepared by repeated immunization of New Zealand White (NZW) rabbits with recombinant human TNF emulsified in complete Freund's adjuvant (DIFCO, IL., USA).

The results indicated that human peripheral blood monocytes are exquisitely sensitive to bacterial endotoxin. Nanogram or even picogram quantities of LPS stimulated high levels of TNF production as well as for IL-1 production.

The results of the effects of compounds on the in vitro TNF production by human monocytes are reported in Table B. The compounds wherein the phenyl substituent group of the pyrrolo[2,1-b]imidazoles and the dihydroimidazo[2,1-a]thiazoles of the present invention are not a sulfinyl derivatives, are potent inhibitors of in vitro TNF production by human monocytes. The compounds wherein the substituent is a sulfinyl derivatives are not active in the in vitro assay. However, the sulfinyl derivatives function as prodrugs to their corresponding sulfide, i.e., they function in vivo to inhibit the production of TNF, because they are metabolized so that they are reductively converted, in vivo, to their corresponding biologically active alkylthio or alkenylthio form. Proof of this in vivo conversion of the sulfinyl derivatives to a biologically active form is indicated by the in vivo activity of the sulfinyl compounds in the in vivo assay described in Utility Models Examples A and B for Compound 2. The conversion of the sulfinyl/sulfoxy pro-drug derivatives to their active sulfinyl structure also holds true for IL-1 activity and is described in detail in U.S. patent application Bender et al., U.S.S.N. 07/365,349, filed June 13, 1989, and in Bender et al., PCT Application, number unknown, Attorneys Docket Number SKB 14446-1, filed contemporaneously herewith,

The results of the effects of compounds of Formula (I) on the in vitro TNF production by human monocytes are reported in Table B.

30

TABLE B

LPS induced TNF Human Monocyte data

<u>Compound No.</u>	<u>IC₅₀ (mM)</u>
1	0.5
35 2 (Sulfoxy derivative)	NA
3 (Sulfinyl derivative)	1.0
4	0.8
5	1.0

TABLE B

LPS induced TNF Human Monocyte data. cont.

	6	1.0
5	7	1.0
	8	1.0
	9	3.0
	10	0.5
	11	0.2
10	12	3.0
	13	0.5
	14	9.0
	15	7.0
	16	7.0
15	NA - not active	

20 The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. Therefore the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way.

CLAIMS

What is claimed is:

5

1. A method of treating a human afflicted a human immunodeficiency virus (HIV), which comprises administering to such human an effective, monokine activity interfering amount of a monokine activity interfering agent.

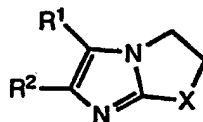
10

2. The method of Claim 1 wherein the monokine activity interfering agent inhibits the production of interleukin-1.

3. The method of Claim 1 wherein the monokine activity interfering agent inhibits the production of TNF.

4. The method of Claim 2 wherein the monokine activity interfering agent is a compound of the formula:

15



wherein:

One of R¹ and R² must be 4-pyridyl and the other is selected from monohalosubstituted phenyl wherein said substituent is selected from halo or C₁₋₄ alkoxy;

20

X is CH₂, CH₂CH₂ or S(O)_n; and

n is 0, 1 or 2;

or a pharmaceutically acceptable salt thereof.

25

5 The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-fluorophenyl; X is S(O)_n and n is 0.

6. The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-fluorophenyl; X is S(O)_n and n is 1.

7. The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-fluorophenyl; X is S(O)_n and n is 2.

30

8. The method of Claim 3 wherein R¹ is 4-fluorophenyl; R² is 4-pyridyl; X is S(O)_n and n is 0.

9. The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-fluorophenyl; and X is CH₂.

35

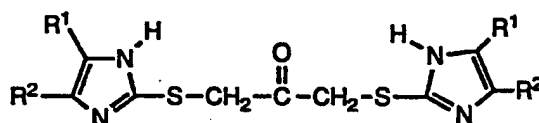
10. The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-methoxyphenyl; X is S(O)_n and n is 0.

-30-

11. The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-methoxyphenyl; and X is CH₂.

12. The method of Claim 3 wherein R¹ is 4-pyridyl; R² is 4-methoxyphenyl; and X is CH₂CH₂.

5 13. The method of Claim 2 wherein the monokine activity interfering agent is selected from a compound of the formula:

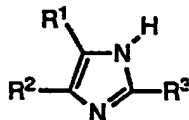


wherein:

10 one of R¹ and R² is 4-pyridyl and the other is monohalosubstituted phenyl;
or a pharmaceutically acceptable salt thereof.

14. The method of Claim 12 wherein R¹ is 4-pyridyl and R² is 4-fluorophenyl.

15 15. The method of Claim 2 wherein the monokine activity interfering agent is a compound of the formula:

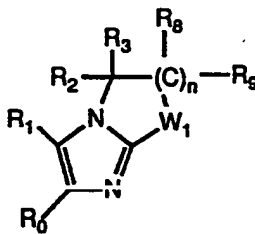


wherein:

20 One of R¹ and R² is 4-pyridyl and the other is selected from monohalosubstituted phenyl; and
R³ is S or SCF₂CF₂H;
or a pharmaceutically acceptable salt thereof.

25 16. The method of Claim 14 wherein R² is 4-pyridyl, R¹ is 4-fluorophenyl and R³ is either SH or SCF₂SF₂H.

17. The method of Claim 2 wherein the monokine activity interfering agent is selected from a compound of the formula:



30 wherein:

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W_1 is $-(CR_4R_5)-(CR_6R_7)-$, $-CR_5=CR_7-$, $-N=CR_7-$, $-S(O)_m-$ or $-O-$;
one of R_1 and R_0 is 4-pyridyl or C_{1-4} alkyl-4-pyridyl, provided that

when R_1 is C_{1-4} alkyl-4-pyridyl the alkyl substituent is located at the 2-position of the pyridine ring, and the other of R_1 and R_0 is

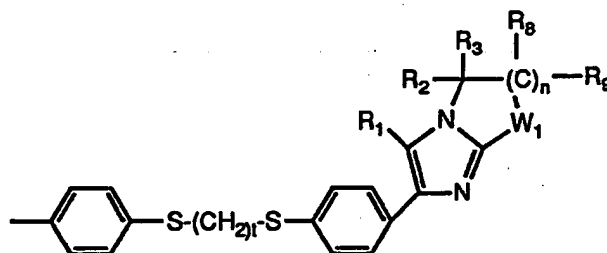
5 (a) phenyl or monosubstituted phenyl wherein said substituent is C_{1-3} alkylthio, C_{1-3} alkylsulfinyl, C_{2-5} 1-alkenyl-1-thio, C_{2-5} 1-alkenyl-1-sulfinyl, C_{3-5} 2-alkenyl-1-thio, C_{3-5} 2-alkenyl-1-sulfinyl, 1-acyloxy-1-alkylthio, C_{1-2} alkoxy, halo, C_{1-4} alkyl or Z wherein Z is $-S-S-Z_1$ and Z_1 is phenyl or C_{1-9} alkyl; or

10 (b) disubstituted phenyl wherein said substituents are, independently, C_{1-3} alkylthio, C_{1-2} alkoxy, halo or C_{1-4} alkyl; or

(c) disubstituted phenyl wherein one of said substituents is C_{1-3} alkylsulfinyl, C_{2-5} 1-alkenyl-1-thio, C_{2-5} 1-alkenyl-1-sulfinyl, C_{3-5} 2-alkenyl-1-thio, C_{3-5} 2-alkenyl-1-sulfinyl or 1-acyloxy-1-alkylthio and the other is C_{1-2} alkoxy, halo, or C_{1-4} alkyl; or

(d) disubstituted phenyl wherein the substituents are the same and are C_{1-3} alkylsulfinyl, C_{2-5} 1-alkenyl-1-thio, C_{2-5} 1-alkenyl-1-sulfinyl, C_{3-5} 2-alkenyl-1-thio, C_{3-5} 2-alkenyl-1-sulfinyl or 1-acyloxy-1-alkylthio or wherein the substituents together form a methylene dioxy group;

(e) monosubstituted phenyl wherein the substituent is



25 t is 0 or 1; W_1 , R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 and R_9 are as defined above;

provided that:

(1.) when W_1 is $-(CR_4R_5)-(CR_6R_7)-$ then

30 n is 0 or 1; and
 R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , and R_9 are, independently, $-H$ or C_{1-2} alkyl; and

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- when R_1 or R_0 is 4-pyridyl, the other of R_1 and R_0 is other than mono- C_{1-2} alkoxy-substituted phenyl or mono-halo-substituted phenyl; or
- when n is 0, R_4 and R_5 together form an oxo; R_4 and R_5 are both fluoro, or one of R_4 and R_5 is H and the other OH; or
- 5 (2.) when W_1 is $-CR_5=CR_7-$ or $-N=CR_7-$ then
- n is 1;
- R_3 , R_5 , R_7 and R_9 are, independently, -H or C_{1-2} alkyl; and
- R_2 and R_8 together represent a double bond in the B ring such that the
- 10 B ring is an aromatic pyridine or pyrimidine ring;
- (3.) when W_1 is $S(O)_m$ then
- m is 0, 1 or 2;
- n is 1 or 2; and
- R_3 and R_9 are, independently, -H or C_{1-2} alkyl;
- 15 R_2 and R_8 are, independently, -H or C_{1-2} alkyl or R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;
- further provided that:
- (a) when R_2 and R_8 are, independently, -H or C_{1-2} alkyl and R_1 or R_0 is 4-pyridyl, then the other of R_1 and R_0 is other than mono- C_{1-2} alkoxy-substituted phenyl or mono-halo-substituted phenyl; and
- 20 (b) when R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring,
- 25 then m is 0 and n is 1; and
- (4) when W_1 is -O- then
- n is 1;
- R_3 and R_9 are, independently, -H or C_{1-2} alkyl; and
- R_2 and R_8 together represent a double bond in the B ring such that the
- 30 B ring is an aromatic oxazole ring;
- or a pharmaceutically acceptable salt thereof.
18. The method of Claim 17 wherein:
- W_1 is $-(CR_4R_5)-(CR_6R_7)-$, $-CR_5=CR_7-$, or $-S(O)_m-$;
- 35 one of R_1 and R_0 is 4-pyridyl or C_{1-2} alkyl-4-pyridyl, provided that when R_1 is C_{1-2} alkyl-4-pyridyl the alkyl substituent is located at the 2-position of the pyridine ring, and the other of R_1 and R_0 is

-33-

(a) monosubstituted phenyl wherein said substituent is C₁₋₂ alkylthio, C₁₋₂ alkylsulfinyl, 1-acyloxy-1-alkylthio, C₁₋₂ alkoxy or halo, or

(b) disubstituted phenyl wherein said substituents are, independently, C₁₋₂ alkylthio or C₁₋₂ alkoxy, or

(c) disubstituted phenyl wherein one of said substituents is C₁₋₂ alkylsulfinyl or 1-acyloxy-1-alkylthio and the other is C₁₋₂ alkoxy, or

(d) disubstituted phenyl wherein the substituents are the same and are C₁₋₂ alkylsulfinyl or 1-acyloxy-1-alkylthio or wherein the substituents together form a methylene dioxy group;

provided that:

(1.) when W₁ is -(CR₄R₅)-(CR₆R₇)- then

n is 0 or 1; and

R₂, R₃, R₄, R₅, R₆, R₇, R₈, and R₉ are -H; and

when R₁ or R₀ is 4-pyridyl, the other of R₁ and R₀ is other than mono-C₁₋₂ alkoxy-substituted phenyl or mono-halo-substituted phenyl;

(2.) when W₁ is -CR₅=CR₇- then

n is 1;

R₃, R₅, R₇ and R₉ are -H; and

R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic pyridine ring;

(3.) when W₁ is S(O)_m then

m is 0, 1 or 2;

n is 1 or 2; and

R₃ and R₉ are -H;

R₂ and R₈ are -H or R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;

further provided that:

(a) when R₂ and R₈ are -H and R₁ or R₀ is 4-pyridyl, then the other of R₁ and R₀ is other than mono-C₁₋₂ alkoxy-substituted phenyl or mono-halo-substituted phenyl; and

(b) when R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring, then m is 0 and n is 1; and

(4) when W₁ is -O- then

-34-

n is 1;

R₃ and R₉ are -H; andR₂ and R₈ together represent a double bond in the B ring such that the

B ring is an aromatic oxazole ring;

5 or a pharmaceutically acceptable salt thereof.

19. The method of Claim 18 wherein the compound is:

2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]imidazole;1 0 2-(4-methylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]imidazole;2-(4-ethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]imidazole;1 5 2-(4-ethylsulfinylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]imidazole;2-(4-methylthiophenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-
pyrrolo[1,2-a]imidazole;2-(4-methylsulfinylphenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-
pyrrolo[1,2-a]imidazole;

2 0 2-(4-methoxyphenyl)-3-(4-pyridyl)-imidazo[1,2-a]-pyridine;

5-(3,4-(methylenedioxy)phenyl)-6-(4-pyridyl)-2,3-dihydroimidazo-
[2,1-b]thiazole;2-(4-methoxyphenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-[5H]-
pyrrolo[1,2-a]imidazole;2 5 2-(4-acetoxymethylthiophenyl)-3-(4-(2-methyl)pyridyl)-6,7-dihydro-
[5H]-pyrrolo[1,2-a]imidazole.2-(trimethylacetylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]imidazole;3 0 6-(4-methylthiophenyl)-5-(4-pyridyl)-2,3-dihydro-imidazo-[2,1-b]-
thiazole;5-(4-methylthiophenyl)-6-(4-pyridyl)-2,3-dihydro-imidazo-
[2,1-b]thiazole;3-(4-methylthiophenyl)-2-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]imidazole;3 5 2-(4-propylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-
[1,2-a]-imidazole;2-(4-methylthiophenyl)-3-(4-(2-ethyl)pyridyl)-6,7-dihydro-[5H]-
pyrrolo-[1,2-a]-imidazole;

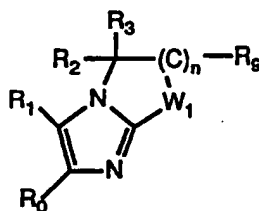
2-(4-Mercaptophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole disulfide;

2-(4-Methoxyphenyl)-3-(4-pyridyl)-7-oxo-5,6-dihydro-[7H]-pyrrolo-[1,2-a]-imidazole;

5 5,6-dihydro-2-(4-Methoxyphenyl)-3-(4-pyridyl)-[7H]-pyrrolo-[1,2-a]-imidazole-7-ol; or

5,6-dihydro-7,7-difluoro-2-(4-Methoxyphenyl)-3-(4-pyridyl)-[7H]-pyrrolo-[1,2-a]-imidazole.

10 20. The method of Claim 3 wherein the monokine activity interfering agent is selected from a compound of Formula (V):



15 wherein:

W₁ is -(CR₄R₅)-(CR₆R₇)-, -CR₅=CR₇-, -N=CR₇-, -S(O)_m- or -O-;
one of R₁ and R₀ is 4-pyridyl or C₁₋₄ alkyl-4-pyridyl, provided that
when R₁ is C₁₋₄ alkyl-4-pyridyl the alkyl substituent is located at the 2-position of
the pyridine ring, and the other of R₁ and R₀ is

20 (a) phenyl or monosubstituted phenyl wherein said
substituent is C₁₋₃ alkylthio, C₁₋₃ alkylsulfinyl, C₂₋₅ 1-alkenyl-1-thio,
C₂₋₅ 1-alkenyl-1-sulfinyl, C₃₋₅ 2-alkenyl-1-thio, C₃₋₅ 2-alkenyl-1-
sulfinyl, 1-acyloxy-1-alkylthio, C₁₋₂ alkoxy, halo, C₁₋₄ alkyl or Z
wherein Z is -S-S-Z₁ and Z₁ is phenyl or C₁₋₉ alkyl; or

25 (b) disubstituted phenyl wherein said substituents are,
independently, C₁₋₃ alkylthio, C₁₋₂ alkoxy, halo or C₁₋₄ alkyl; or

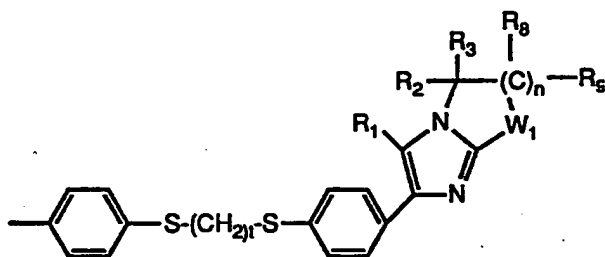
(c) disubstituted phenyl wherein one of said substituents is
C₁₋₃ alkylsulfinyl, C₂₋₅ 1-alkenyl-1-thio, C₂₋₅ 1-alkenyl-1-sulfinyl,
C₃₋₅ 2-alkenyl-1-thio, C₃₋₅ 2-alkenyl-1-sulfinyl or 1-acyloxy-1-
30 alkylthio and the other is C₁₋₂ alkoxy, halo, or C₁₋₄
alkyl; or

(d) disubstituted phenyl wherein the substituents are the same
and are C₁₋₃ alkylsulfinyl, C₂₋₅ 1-alkenyl-1-thio, C₂₋₅ 1-alkenyl-1-
sulfinyl, C₃₋₅ 2-alkenyl-1-thio, C₃₋₅ 2-alkenyl-1-sulfinyl or 1-

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acyloxy-1-alkylthio or wherein the substituents together form a methylene di xy group;

(e) monosubstituted phenyl wherein the substituent is



t is 0 or 1; W_1 , R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 and R_9 are as defined above;

provided that:

(1.) when W_1 is $-(CR_4R_5)-(CR_6R_7)-$ then

n is 0; R_4 and R_5 may together form an oxo; R_4 and R_5 are both fluoro; or one of R_4 and R_5 is H and the other OH; or

(2.) when W_1 is $-CR_5=CR_7-$ or $-N=CR_7-$ then

n is 1;

R_3 , R_5 , R_7 and R_9 are, independently, -H or C_{1-2} alkyl; and

R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic pyridine or pyrimidine ring;

(3.) when W_1 is $S(O)_m$ then

m is 0, 1 or 2;

n is 1 or 2; and

R_3 and R_9 are, independently, -H or C_{1-2} alkyl;

R_2 and R_8 are, independently, -H or C_{1-2} alkyl or R_2 and R_8

together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;

further provided that:

(a) when R_2 and R_8 are, independently, -H or C_{1-2} alkyl and R_1 or R_9 is 4-pyridyl, then the other of R_1 and R_9 is other a mono-fluoro-substituted phenyl; and

(b) when R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring, then m is 0 and n is 1; and

(4) when W_1 is -O- then

n is 1;

R_3 and R_9 are, independently, -H or C_{1-2} alkyl; and

R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic oxazole ring;
or a pharmaceutically acceptable salt thereof.

5 21. The Method of Claim 20 wherein

W_1 is $-(CR_4R_5)-(CR_6R_7)-$, $-CR_5=CR_7-$, or $-S(O)_m-$;

one of R_1 and R_0 is 4-pyridyl or C_{1-2} alkyl-4-pyridyl,

provided that when R_1 is C_{1-2} alkyl-4-pyridyl the alkyl substituent is located at the 2-position of the pyridine ring, and the other of R_1 and R_0 is

10

(a) monosubstituted phenyl wherein said substituent is C_{1-2} alkylthio, C_{1-2} alkylsulfinyl, 1-acyloxy-1-alkylthio, C_{1-2} alkoxy or halo, or

15

(b) disubstituted phenyl wherein said substituents are, independently, C_{1-2} alkylthio or C_{1-2} alkoxy, or

(c) disubstituted phenyl wherein one of said substituents is C_{1-2} alkylsulfinyl or 1-acyloxy-1-alkylthio and the other is C_{1-2} alkoxy, or

20

(d) disubstituted phenyl wherein the substituents are the same and are C_{1-2} alkylsulfinyl or 1-acyloxy-1-alkylthio or wherein the substituents together form a methylene dioxy group;

provided that:

(1.) when W_1 is $-CR_5=CR_7-$ then

n is 1;

25

R_3 , R_5 , R_7 and R_9 are $-H$; and

R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic pyridine ring;

(2.) when W_1 is $S(O)_m$ then

m is 0, 1 or 2;

30

n is 1 or 2; and

R_3 and R_9 are $-H$;

R_2 and R_8 are $-H$ or R_2 and R_8 together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring;

further provided that:

35

(a) when R_2 and R_8 are $-H$ and R_1 or R_0 is 4-pyridyl, then the other of R_1 and R_0 is other than mono-fluoro-substituted phenyl; and

-38-

(b) when R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic thiazole ring, then m is 0 and n is 1; and

(4) when W₁ is -O- then

5

n is 1;

R₃ and R₉ are -H; and

R₂ and R₈ together represent a double bond in the B ring such that the B ring is an aromatic oxazole ring;

or the pharmaceutically acceptable salts thereof.

10

22. The method of Claim 20 wherein the compound is

2-Phenyl-3-pyridyl-6,7-dihydro-[5H]-pyrrolo-[1,2-a]imidazole;

2-4-Bromophenyl-3-pyridyl-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;

2-(4-Pyridyl)-3-(4-fluorophenyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]imidazole;

15

2-(4-Fluorophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;

2-(4-Methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;

20

2-(4-Pyridyl)-3-(4-methylthiophenyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole;

2-(4-Methylthiophenyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole;

2-(4-Methoxyphenyl)-3-(4-pyridyl)-7-oxo-5,6-dihydro-7H-pyrrolo-[1,2-a]imidazole;

25

5,6-Dihydro-2-(4-methoxyphenyl)-3-(4-pyridinyl)-[7H]-pyrrolo[1,2-a]-imidazole-7-ol;

2-(4-Acetoxymethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro[5H]pyrrolo-[1,2-a]-imidazole;

30

2-(4-Methoxyphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]imidazole;

6-(4-Fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole;

6-(4-Fluorophenyl)-5-(4'-pyridyl)-2,3-dihydroimidazo-[2,1-b]thiazole-1-oxide;

35

6-(4-Fluorophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole-1,1-dioxide;

5-(4-Fluorophenyl)-6-(4'-pyridyl)-2,3-dihydroimidazo-[2,1-b]thiazole;

2-(4-Methylsulfoxyphenyl)-3-(4-pyridyl)-6,7-dihydro[5H]-pyrrolo-

[1,2-a]imidazole;

2-(4-Ethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole; or

5 2-(4-Ethylsulfonophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole.

23. A method of treating HIV infection in a human afflicted with human immunodeficiency virus (HIV) infection which comprises administering to such human an effective amount of a compound known as 5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo-(2,1-b)-thiazole.

24. A method of treating HIV infection in a human afflicted with human immunodeficiency virus (HIV) infection which comprises administering to such human an effective amount of a compound known as 2-(4-methylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]-imidazole.

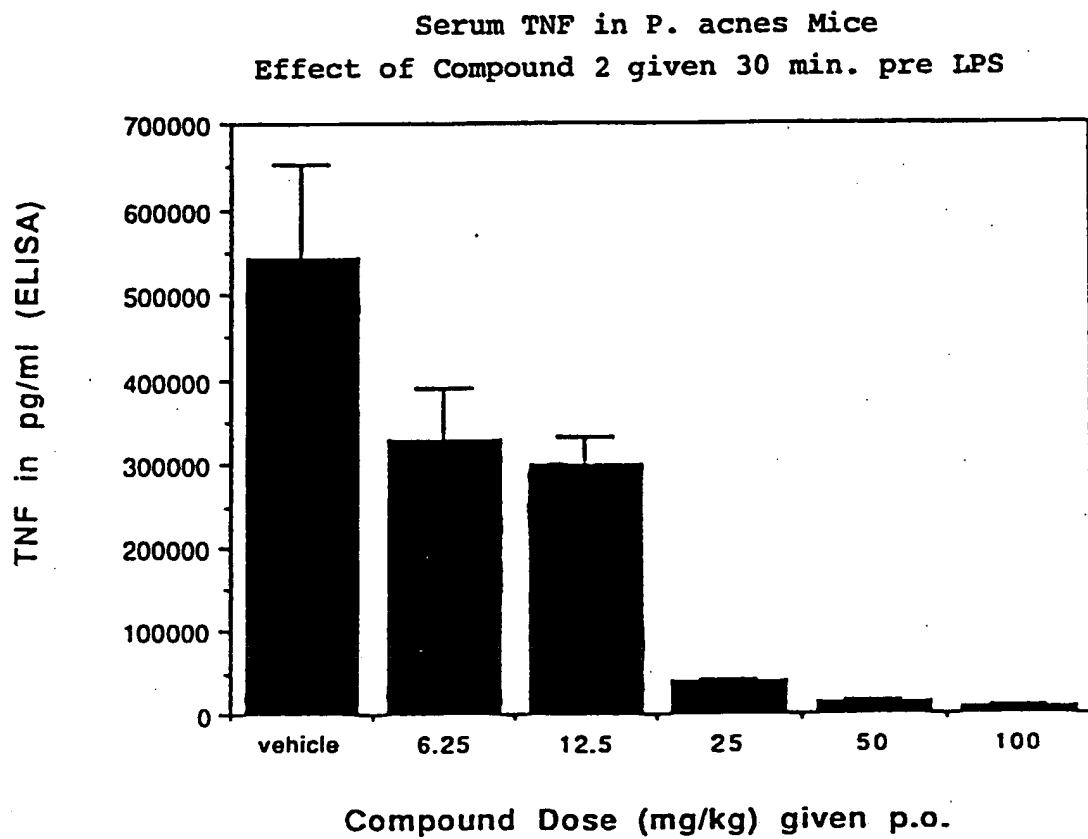
25. A method of treating HIV infection in a human afflicted with human immunodeficiency virus (HIV) infection which comprises administering to such human an effective amount of a compound known as 2-(4-Ethylthiophenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole.

26. A method of treating HIV infection in a human afflicted with human immunodeficiency virus (HIV) infection which comprises administering to such human an effective amount of a compound known as 2-(4-Methylthiophenyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro-[5H]-pyrrolo-[1,2-a]-imidazole.

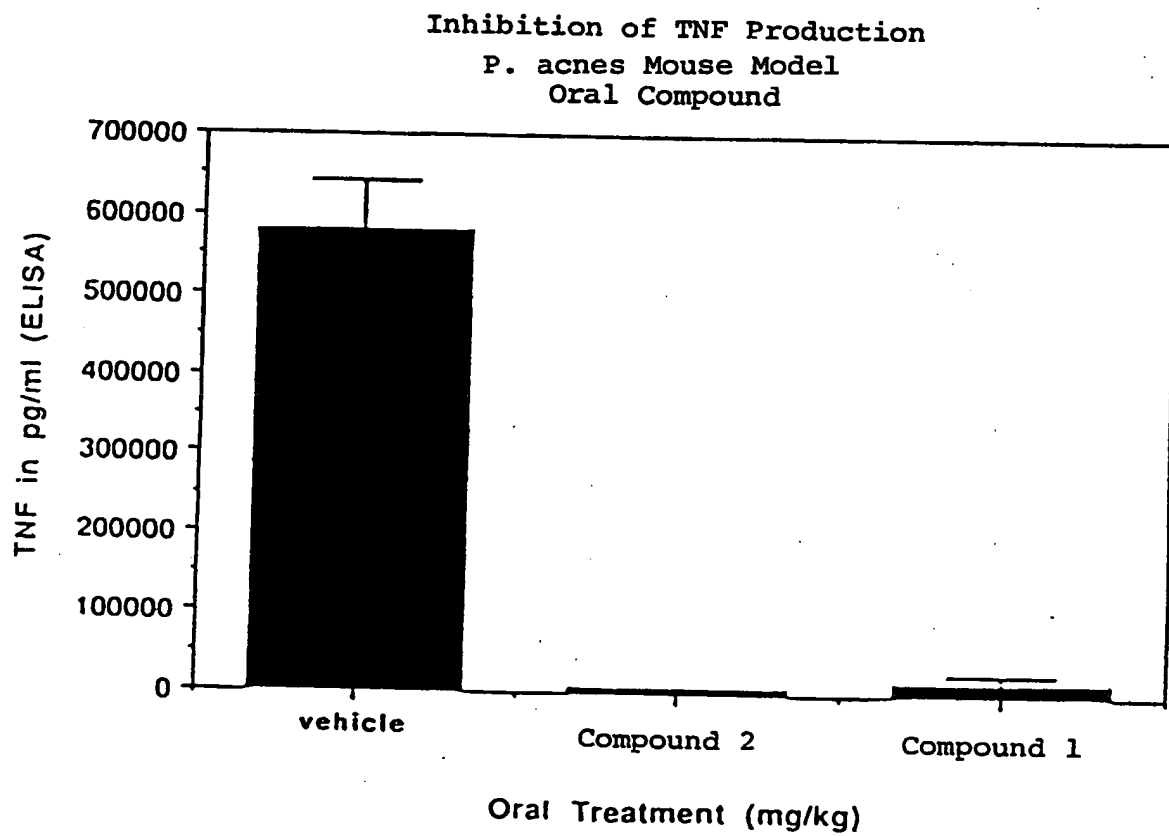
27. A method of treating HIV infection in a human afflicted with human immunodeficiency virus (HIV) infection which comprises administering to such human an effective amount of a compound known as 6-(4-Fluorophenyl)-5-(4-pyridyl)-2,3-dihydroimidazo[2,1-b]thiazole-1,1-dioxide.

28. The method of any of Claims 23 to 27 wherein the compound is administered orally, parenterally, by inhalation or topically.

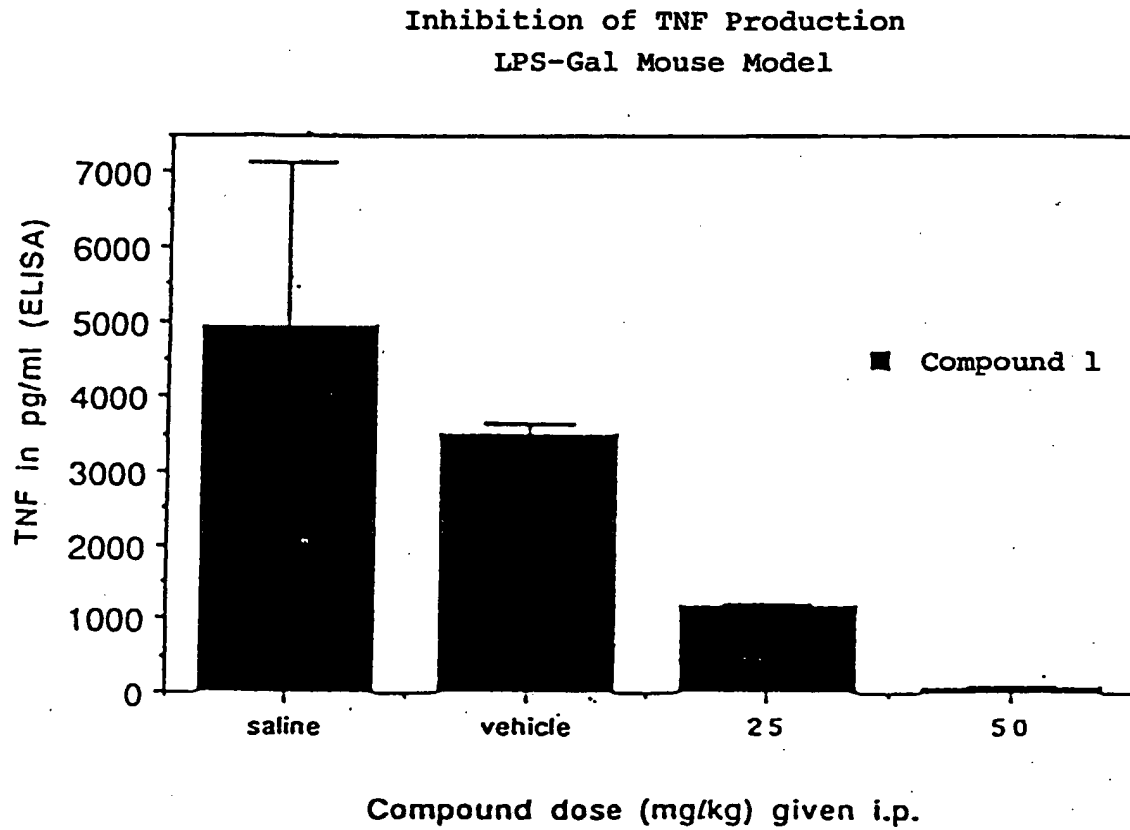
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Figure 1



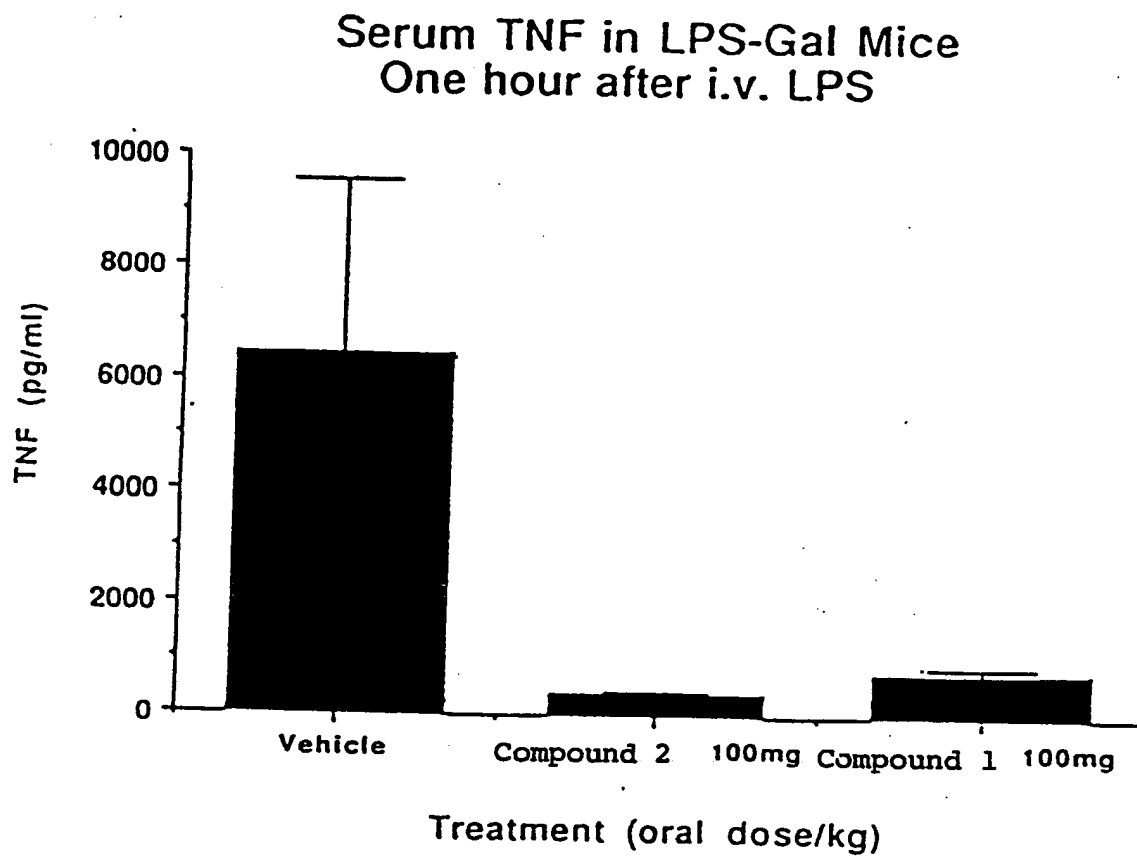
2/5
Figure 2

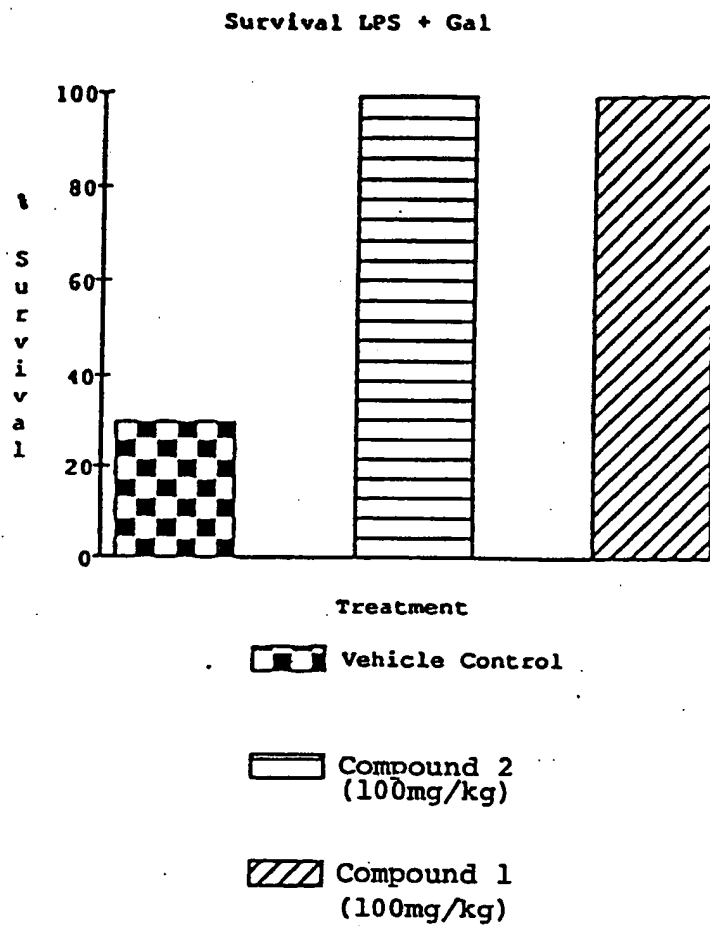


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Figure 3



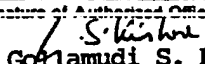
4/5
Figure 4



5/5
Figure 5

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/03380**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC <div style="text-align: center;"> IPC (5): A01N 43/06, 43/40; A61K 31/415 U.S. Cl. 514/338, 387 </div>						
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;"> Minimum Documentation Searched ⁷ </div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">Classification System</th> <th style="width: 80%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: middle;">U.S.</td> <td style="text-align: center; vertical-align: middle;">514/333, 338, 387, 885</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div>			Classification System	Classification Symbols	U.S.	514/333, 338, 387, 885
Classification System	Classification Symbols					
U.S.	514/333, 338, 387, 885					
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹						
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³				
Y	US, A, 4,794,114 (BENDER) 27 DECEMBER 1988 See abstract, column 6, lines 25-46, column 7, lines 27-31, column 8, lines 32-34 and column 9, lines 2-29.	4-12 & 17-28				
Y	The Journal of Immunology, Vol. 142, 15 JANUARY 1989 (CLOUSE) pages 431-438; See abstract.	1-3				
Y	EP, A, 0 230 574 (YALE UNIVERSITY) 05 AUGUST 1987 See page 2, lines 4-7.	1-3				
Y	Proceedings of the National Academy of Science, USA, (OSBORN) Vol. 86, April 1989, pages 2336-2340; see Abstract.	1-3				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">18 JULY 1990</div>		Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">14 NOV 1990</div>				
International Searching Authority <div style="text-align: center; font-weight: bold;">ISA/US</div>		Signature of Authorized Officer <div style="text-align: center;">  Goklamudi S. Kishore </div>				

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,3}, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this International application as follows:

Species 1 in claims 4-12 and 17-28.
 Species 2 in claims 13 and 14.
 Species 3 in claims 15 and 16.

(see attachment)
 Telephone Practice

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Claims 1-3 and Species 1 in claims 4-12 and 17-28.
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

(Con't. Supplement Sheet)

Species lack unity of invention since they do not form single invention concept as required by PCT Rule 13. Species are patentably distinct.

WEST☐ Generate Collection

L5: Entry 2 of 3

File: PGPB

PGPUB-DOCUMENT-NUMBER: 20020068057
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020068057 A1

o d p
w
2nd ref
Jun 6, 2002

TITLE: TREATMENT OF AUTOIMMUNE AND INFLAMMATORY DISORDERS

PUBLICATION-DATE: June 6, 2002

08/617,734

INVENTOR-INFORMATION:

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WILLIAMS, RICHARD O.	LONDON		GB	

US-CL-CURRENT: 424/133.1; 424/141.1, 424/152.1, 424/172.1, 514/12, 514/8

CLAIMS:

What is claimed is:

1. A method of treating autoimmune or inflammatory disease in a mammal comprising administering to said mammal a therapeutically effective amount of a combination of a CD4+ T cell inhibiting agent and a TNF antagonist.
2. A method of claim 1, wherein the CD4+ T cell inhibiting agent is administered simultaneously with the TNF antagonist.
3. A method of claim 1, wherein the CD4+ T cell inhibiting agent is administered sequentially with the TNF antagonist.
4. A method of claim 1, wherein the CD4+ T cell inhibiting agent and the TNF antagonist are administered by a route selected from the group consisting of: subcutaneously, intravenously, and intramuscularly.
5. A method of claim 1, wherein the CD4+ T cell inhibiting agent and the TNF antagonist are administered in a pharmaceutically acceptable vehicle.
6. A method of claim 1, wherein an anti-inflammatory agent is administered in conjunction with the CD4+ T cell inhibiting agent and the TNF antagonist.
7. A method of claim 6, wherein the anti-inflammatory agent is an agent interfering with the activity or synthesis of TNF.
8. A method of claim 6, wherein the anti-inflammatory agent is an agent interfering with the activity or synthesis of IL-1.
9. A method of claim 6, wherein the anti-inflammatory agent is an agent interfering with the activity or synthesis of IL-6.
10. A method of claim 6, wherein the anti-inflammatory agent is a cytokine with anti-inflammatory properties.